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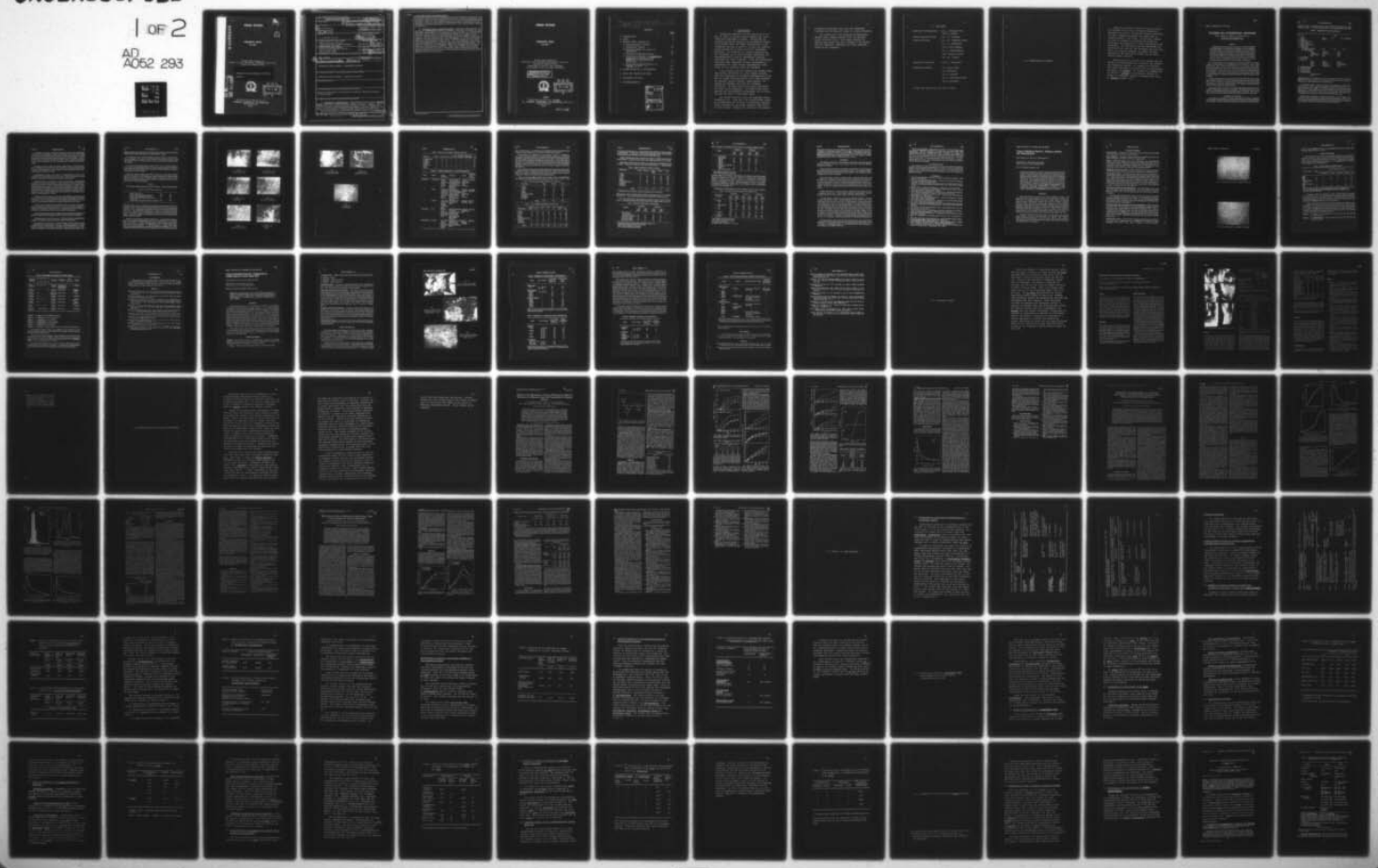

HUMAN MYCOSES

(1)

CONSOLIDATED REPORT
1971-1977

Research Project sponsored by
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

a. Immunology of dermatomycoses: Experimental infection with T. mentagrophytes was produced in guinea pigs by cutaneous scarification method. The infection lasted for 40 days with complete clinical recovery. Repeated infections to the same animals revealed the development of acquired immunity as evidenced by rapid healing and increased dermal sensitivity of dealyed type.

Active immunization of guinea pigs with soluble extract of T. mentagrophytes

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in Freund's complete adjuvant revealed the presence of humoral antibodies, viz., precipitin (titer of 1:16), agglutinin (titer of 1:1024) and complement-fixing antibodies (1:10) and cell-mediated immune mechanisms as evidenced by the development of delayed hypersensitivity reactions to the soluble extract injected intradermally.

b. Molecular basis of miconazole action: Miconazole labilizes rate liver lysosomes. Its labilizing effect is followed by measuring the release of lysosomal hydrolases, namely, acid phosphatase, β-glucuronidase, and arylsalfatase. The effect of miconazole is concentration dependent in the range of 10^{-5} to $1.2X10^{-4}$. However, at higher concentrations, miconazole inhibits enzyme release but does not inhibit enzyme activities per se. The effect of miconazole depends on the drug/lysosome ratio and is influenced by the pH of the incubation media, being minimal at alkaline pH. Some of the membrane active drugs have been compared with miconazole for their lysosome-labilizing action. The effect of miconazole on the lysosomal membrane is confirmed by a decrease in turbidity of the lysosomal suspension. Thus, these studies and our earlier work on the mechanism of action of miconazole clearly reveal that the drug interacts with both cellular systems and subcellular systems and impairs their membrane structure and function.

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This report contains reports and reprints of journal articles produced for this contract on the following topics: YGK

CONTENTS

A. INTRODUCTION

Increasing importance is now being given to the role of fungi in causing various systemic and superficial skin disorders in humans. Particularly in countries located in tropical and subtropical regions of the world, where the fungal contamination of the atmosphere and soil is known to be high, the possible health hazards due to fungi have to be considered seriously. The situation is further accentuated by the widespread use of broad spectrum antibiotics and immunosuppressive drugs in controlling bacterial infections and other disorders. It is well established that some fungi considered as mere contaminants or saprophytes can, under altered physiological conditions, be the causative agents for diseases.

A few surveys conducted earlier have clearly shown that mycotic infections are fairly common in India. Hence the importance of detailed investigations in the area of fungal infections needs to be hardly stressed. The research project entitled 'Human Mycoses' was started in September 1971 with the following objectives: to study superficial mycotic infections and their causal organisms; to examine their pathogenicity and host-parasite relationship, and to study the mode of action of antifungal drugs.

The results achieved during the programme period (September 1971 - June 1977) have significantly helped in understanding the etiology of superficial mycoses, their pathogenicity and immune responses they elicit in host animals. Besides, the efficacy of miconazole,

a synthetic antifungal drug, has been clinically evaluated and its molecular basis of action elucidated.

The results have been presented in Half-yearly reports (six), Annual reports (five) and Progress Report Abstracts (two). This report wherein the results from recent work and a summary of the earlier work are presented, represents the final technical report of the programme.

B. S T A F F

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C.1. MICROBIOLOGICAL ASPECTS

Among the infective diseases of man and animals the diseases due to fungi are widely distributed throughout the world. Superficial mycotic infections are more commonly encountered in tropics than in temperate parts of the world. The tropical climate of India is supposed to influence and favour the occurrence and maintenance of these infections in the population. The superficial mycotic infections are confined to the skin and its appendages like nail and hair. The clinical symptoms are highly variable depending on the individual factors and the etiological agents involved.

Studies have been carried out to examine the incidence of dermatomycoses and to isolate and identify the etiological agents. The studies have revealed that these infections are caused both by dermatophytes and species of Candida. The studies on pathogenicity of species of Candida in experimental animals revealed the pathogenic ability of species other than C. albicans (pages, 5-25).

Arogya—J. Health Sci., 1977, *III*, 31-40

STUDIES ON SUPERFICIAL MYCOSES

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ABSTRACT

India being a tropical country, most of its parts are under the influence of sustained periods of combined heat and humidity, and it is supposed to have high incidence of mycotic infections. Studies carried out in different parts of the country on the incidence of dermatomycoses and their causal organisms have rendered varied findings. In the present study 374 patients with suspected superficial mycotic infections were examined. The etiological agents of these infections were isolated from the lesions and characterized as to their species. Susceptibility of these causal organisms to various antifungal agents generally used in clinical practice was determined.

Ever since Sabouraud's classic publication, 'Les Teignes' appeared, the widespread occurrence of dermatophytes is well established from the findings of various workers throughout the world. However, dermatomycoses are more common in the tropics than in the temperate parts of the world. India being a tropical country, situated within the tropical and subtropical belts of the world, is supposed to have high incidence of mycotic infections. Most parts of India are under the influence of sustained periods of combined heat and humidity recurring annually; and these geographic factors directly or indirectly influence the occurrence of mycotic infections and their maintenance in the population.

The study of dermatomycoses was started in India in early 1920s mainly at the Calcutta School of Tropical Medicine[1]. Since then several such studies have been made in various parts of India. During the last decade, surveys have been carried out on the incidence of dermatomycoses and their causal organisms[2–9]. The findings varied depending upon the parts of the country and population studied.

In the present study, 374 patients with suspected superficial mycotic infections, who attended the dermatological clinics were examined. The etiological agents of these infections were isolated from the lesions, and characterized as to their species. Their susceptibility to various antifungal agents generally used in clinical practice was determined.

MATERIALS AND METHODS

The samples were collected at the Skin Department (Out Patient) of Employees' State Insurance Hospital, Rajajinagar, Bangalore, and Government Hospital, Bellary. A total number of 374 patients suspected of superficial infections were examined over a

period of 2 years. The proforma used to collect the relevant information of the case is shown in Chart 1. The cases were diagnosed as 'tinea' denoting fungus and a suffix

CHART 1 — PROFORMA FOR THE CASE REPORT

E. S. I. No. : Date :

1. Name : Age : Yrs. Sex : Male/Female
2. Address : Religion :
3. Occupation :
4. Duration of the illness :
5. General health :
6. History of animal contact :
7. Family history :
8. Clinical lesions :
   (*a*) Primary site of disease : Skin Nail Hair
   (*b*) Regions affected :

Groin	Buttocks	Axilla
Abdomen	Foot & toes	Cheek
Hands & fingers	Back	Chest
Thigh	Loin	Face

9. No. of lesions :
10. Size of lesions :
11. Characters :

Border	Redness	Scales
Papule	Weaping	Lichenification
Vesicle	Pigmentation	Secondary infection
Pustules	Depigmentation	Itching

12. Provisional diagnosis :
13. Differential diagnosis :
14. Previous treatment :
15. Treatment advised :
16. Laboratory findings :
    Direct examination :
    Culture :

added denoting the site of infection, except in the case of tinea versicolor where versicolor depicts the varied nature of the lesions. Besides skin infections, a case of external ear infection and a vaginal infection were also encountered.

(a) *Collection of samples.* The skin scrapings and nail clippings were collected on a clean paper using a sterile scalpel after cleaning the site with 70% ethyl alcohol. From cases of tinea versicolor, samples were collected according to the method described by Shing Lin Lii[10]. After treating the site with 70% ethyl alcohol, procaine was injected intradermally into the lesions, to form a wheal of 1 cm diameter. The epidermis was then peeled off with a clean new razor blade and collected on sterile paper.

(b) *Microscopic examination.* A sample of the scrapings, and nail clippings were mounted in a drop of *KOH*-glycerine (10% *KOH* and 5% glycerine) on a slide, covered with a cover slip, slightly warmed over the flame and then examined under the microscope for the presence or absence of mycelial bits, spores or yeast-like cells.

(c) *Culturing of organisms.* Sabouraud's glucose agar (S.g. agar) supplemented with antibiotics (chloramphenicol, 0.004%, cycloheximide, 0.05%) and Littman Oxgall agar were used to isolate dermatophytes and yeasts. Some of the scrapings were spread on agar slants and the remaining scrapings were placed in sterile petri plates. S.g. agar (at 40–45 C) was poured into these plates and gently swirled to disperse the scrapings evently. Plates were incubated at room temperature, and examined at intervals for a month.

In addition to the above media, Martin-Scott's medium[11](sodium tauroglycocholate, 10%; oxopeptone, 5%; agar, 2%) was used for isolation of causal organism from tinea versicolor. The epidermal pieces were inoculated on slants, incubated at 25C and 37C and examined at intervals for a month.

(d) *Identification of isolates.* The isolates were identified based on their colony morphology and then subcultured on S.g. agar slants. Further characterization of these isolates was done by their microscopic and biochemical characteristics.

(e) *Characterization of dermatophytes.* Species of *Trichophyton, Epidermophyton* and *Microsporum* were characterized by their colony morphology, viz.,shape, pigmentation, and presence or absence of micro-and macroconidia, chlamydospores, racquet, pectinate and spiral hyphae, on microscopy. Production of macroconidia in *T. rubrum* was confirmed using beef heart infusion tryptose agar (Difco Laboratories, London). Peptone broth (peptone, 10 g; distilled water 100 ml, *pH* 6.4) containing bromothymol blue as indicator was used to study and differentiate growth properties of dermatophytes particularly *T. mentagrophytes* and *T. rubrum.*

Christensen's urea agar[13] (peptone, 1.0 g; NaCl, 5.0 g; KH_2PO_4, 2.0 g; glucose, 5.0 g; agar, 20 g; 6 ml of 0.2% phenol red solution in 50% ethanol and distilled water, 1000 ml) containing 2% urea was inoculated with the dermatophytes and incubated at 27 C for 8 days. Urease production is indicated by change of colour from yellow to pink.

Lab-lemco agar[14] containing Lab-lemco beef extract (Oxoid, London), 2.5 g; glucose, 5.0 g; agar, 20.0 g; and distilled water, 1000 ml was used to examine pigment formation. The *pH* of the medium was adjusted to 6.5–7.5. Inoculated slants were incubated at 27C for 12 to 14 days and the reverse side was examined for pigmentation.

In all the above mentioned studies, standard strains of dermatophytes obtained from School of Tropical Medicine and Hygiene, London, England, were used as reference.

(f) *Characterization of yeast-like organisms.* The characterization and identification of species of *Candida* have been carried out by methods reported earlier[15].

(g) *Preparation of drug solutions.* Nystatin (Sarabhai Chemicals, Baroda, India) was dissolved in sterile distilled water containing 1% dimethyl formamide at a concentration of 1 mg/ml. Stock solution of griseofulvin (Sigma Chemical Co., St. Louis, U.S.A.) was prepared in 50% dimethyl formamide. Miconazole nitrate (Ethnor Ltd.,

Bombay, India) and buclosamide (active ingredient of Zadit, Hoehst Laboratories, Bombay, India) were dissolved in 50% ethanol at a concentration of 1 mg/ml.

(h) *Method of testing.* Serial (two-fold) tube dilution method was used for yeasts. Tubes containing 25, 12.5, 6.25, 3.12, and 1.56 μg/ml of the drug in 5 ml of S.g. broth were inoculated with 1.5×10^5 cells of yeast in 0.1 ml of inoculum and incubated at 30 C for 24–48 hr.

S.g. broth tubes containing drugs at 50, 40, 30, 25, 15, 5, 2.5, 1.5, and 0.1 μg/ml in a 5 ml system were inoculated with 0.05 ml of the spore/mycelial suspension of dermatophytes (stock suspension adjusted to 85% transmission in Klett–Summerson colorimeter) and incubated at 30 C for 14 days.

Minimum inhibitory concentrations (MIC) of the drug to bring about complete inhibition of the growth was determined at the end of 24 hr (yeasts) and 7 days (dermatophytes) and expressed as μg/ml. Minimum fungicidal concentration (MFC) to bring about complete inhibition of the growth and viability was determined at the end of 48 hr (yeasts) and 14 days (dermatophytes).

RESULTS

The incidence of fungi in clinical cases is shown in Table 1. Out of 374 cases suspected

Table 1. *Incidence of fungi in clinical cases*

Number of cases studied	374	%
Number of cases found positive by microscopy	129	34.2
Number of cases found positive by culture	123	32.8
Number of cases found positive either by microscopy or by culture	184	49.2
Number of cases where mixed infections were encountered	14	3.7

of fungal infections, 34.2% were found positive for fungi by direct examination and 32.8% by culture. Large number of patients had multiple lesions and some had even mixed infections (3.7%). In 9 patients, dermatophytes and yeasts were isolated together from same lesions. The dermatophytes isolated were *T. rubrum* (5 cases) and *E. floccosum* (4 cases). In another 5 cases, *T. rubrum* and *T. violaceum* were isolated from the same lesions. In some cases, multiple and clinically distinct lesions in the same patient yielded different fungi upon culture.

Incidence of etiological agents in different clinical types: A large number of dermatophytes and yeasts were isolated mainly from two types of clinical lesions, viz., t.corporis and t. cruris (Table 2). *T. rubrum* constituted the most common etiological agent (31.6%) followed by *E. floccosum* (18.6%). *T. mentagrophytes* and *T. violaceum* were isolated from 12 and 10 cases, respectively. *M. canis* was isolated from a single case of t. cruris. A significantly high percentage of yeast-like organisms (32.3%) were isolated from various

Fig. 1
T. mentagrophytes (×450)
Clusters of microconidia

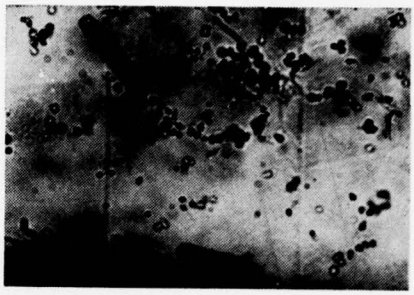

Fig. 2
T. mentagrophytes (×450)
Micro and macroconidia

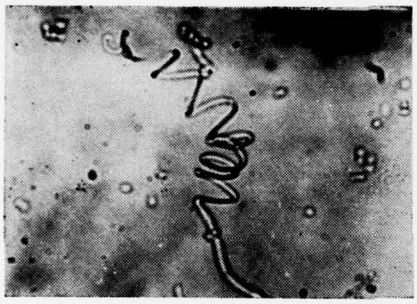

Fig. 3
T. mentagrophytes (×450)
Spiral hyphae

Fig. 4
T. rubrum (×450)
Mycelium with microconidia

Fig. 5
T. rubrum (×450)
Mycelium with macroconidia

Fig. 6
T. violaceum (×100)
Mycelia

Fig. 7
E. floccosum (×450)
Mycelia with macroconidia

Fig. 8
M. canis (×450)
Macro and Microconidia

Fig. 9
M. canis (×360)
Raquet hyphae

Table 2. *Incidence of causal organisms in different clinical types*

Organisms	t. capitis	t. corporis	t. cruris	t. pedis	inter-trigo	Onychia & paronychia	Otomy-coses	Vulvo vaginitis	Total
T. mentagrophytes	1	4	7	...	...	...	...	...	12
T. rubrum	...	25	17	...	1	...	...	...	43
T. violaceum	3	7	...	...	...	...	...	...	10
M. canis	...	...	1	...	...	...	...		1
E. floccosum	...	7	15	1	2	...	...	...	25
Yeasts	2	8	13	4	9	7	...	1	44
A. niger	...	...	...	...	...	...	1	...	1

CHART 2 — CHARACTERIZATION OF THE DERMATOPHYTES

Genus	Species	Colony morphology	Microscopy	Growth	Pigmentation in Lab-lemco agar
Trichophyton	*mentagrophytes*	Granular, flat, few aerial hyphae, reverse yellowish brown	Abundant sessile and oval microconidia in clusters, macroconidia few, cylindrical, 4-5 celled with blunt ends. Spiral hyphae. (Figs. 1-3)	Growth on surface with craters in the centre. Urease + ve.	Pale brown
,,	*rubrum*	Granular with central umbo. Pinkish white surface, a few aerial hyphae, reverse cherry red	Abundant sessile microconidia along the hyphae. Macroconidia absent, produced on beef heart infusion tryptose agar. 4-5 celled, cigar shaped (Figs. 4, 5)	Submerged mycelia, a few as hemispherical ball urease—ve	Pronounced cherry red
,,	*violaceum*	Creamy white or waxy later becomes violet pink, very few aerial hyphae	No spores, mycelia short and stout (Fig. 6)	Submerged fluffy balls	Pale pink colour
Microsporum	*canis*	Yellowish brown granular with brown surface, aerial hyphae present.	Macroconidia abundant on long aerial conidiophore, spindle shaped, 4-5 celled microconidia, oval, sessile, characteristic racquet hyphae (Figs. 8, 9)	Submerged hemispherical balls	Pale brown
Epidermophyton	*floccosum*	Flat brownish powdery colony with radial furrows, no aerial mycelia	Abundant macroconidia either singly or in clusters, 3-5 celled, club shaped intercalary (Fig. 7)	Partially submerged fluffy balls	Pale brown

types of clinical lesions. A detailed account of incidence, isolation and characterization of these yeasts-like organisms as to their species has been reported earlier[15].

Characterization and identification of dermatophytes. Species of *Trichophyton, Microsporum* and *Epidermophyton* were identified and characterized based on their colony morphology, pigmentation, presence or absence of macroconidia, other hyphal structures and some of their biochemical potentialities, as given in Chart 2. Like *T. rubrum* one of the strains of *T. mentagrophytes*, viz., HM 147 produced yellowish red pigment, produced both macro-macro-and microconidia as well. The identity of this isolate was confirmed byproduction of urease in urea agar and inability to produce red pigment in Lab-lemco agar (Chart 2).

No correlation between the types of clinical lesions and the etiological agent isolated is evident. However, *T. violaceum* was always observed in t. capitis cases where lesions were of either single or multiple kerion types, while in the glabrous regions the lesions caused by *T. violaceum* were circinate with pronounced border.

Analysis of cases into different clinical types in relation to age, sex and occupation. The distribution of different clinical types in men and women is given in Table 3. The

Table 3. *Analysis of cases into different clinical types with relation to sex*

Clinical types	Males	Females	Total
t. capitis	8	1	9
t. corporis	73	82	155
t. cruris	94	11	105
t. pedis	20	19	39
intertrigo	6	9	15
onychia and paronychia	14	17	31
t. versicolor	22	8	30
vulvovaginitis	—	1	1
otomycoses	—	1	1
TOTAL	237	149	386

Table 4. *Analysis of different clinical types in relation to the age*

Clinical types	10 yrs and below	11—20 yrs.	21—30 yrs.	31—40 yrs.	41—50 yrs.	51 yrs and above	Total
t. capitis	7	2	—	—	—	—	9
t. corporis	21	18	47	38	23	13	155
t. cruris	2	4	45	31	11	12	105
t. pedis	6	5	11	9	6	2	39
intertrigo	—	2	4	4	3	2	15
onychia and paronychia	—	2	7	8	10	4	31
t. versicolor	—	5	13	10	1	1	30
otomycoses	1	—	—	—	—	—	1
vulvovaginitis	1	—	—	—	—	—	1
TOTAL	38	34	127	100	54	33	386

most common clinical type, viz., t. corporis (40% of the incidence) was observed equally in both the sexes. T. cruris and t. versicolor were found more in man. But in women, the predominant sites of infection are foot, nail and intertrigal regions.

People in the age group of 21 to 40 years were found to be affected more than other groups (Table 4). T. capitis was found only in the younger age groups of 10 to 20 years.

The analysis of clinical types in relation to the occupation of the patients is given in Table 5. Infections of scalp were found only among students. T. corporis though

Table 5. *Analysis of clinical types with relation to occupation*

Clinical types	Students	Housewives	Factory workers	Others*	Total
t. capitis	8	—	1	—	9
t. corporis	19	68	40	28	155
t. cruris	8	9	44	44	105
t. pedis	8	12	9	10	39
intertrigo	—	10	2	3	15
onychia and paronychia	3	16	4	8	31
t. versicolor	6	6	12	6	30
vulvovaginitis	1	—	—	—	1
otomycoses	1	—	—	—	1
TOTAL	54	121	112	99	386

*include office workers, businessmen, artisans and manual labourers of both the sexes.

affected all the classes of people, factory workers and housewives seem to be affected more (28.5%). T. cruris was found more in factory workers than in any other group.

The *in vitro* susceptibility of dermatophytes to antimycotic agents is shown in Tables 6 and 7. Griseofulvin exerted both growth inhibition and fungicidal effect in the con-

Table 6. *Susceptibility of dermatophytes to antifungal agents*

Organisms	Griseofulvin		Miconazole	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
Epidermophyton floccosum HM 358	1.5	1.5	0.5	1.5
Microsporum canis HM 382	5.0	5.0	1.5	1.5
*Microsporum adounii**	1.5	1.5	0.5	1.5
*Microsporum gypseum**	1.5	1.5	1.5	1.5
*Microsporum nanum***	2.5	2.5	1.5	1.5

*Obtained from School of Tropical Medicine & Hygiene, London, U. K.
**Obtained from Willingdon Hospital, New Delhi, India.
MIC —Minimum Inhibilitory Concentration.
MFC—Minimum Fungicidal Concentration.

Table 7. *Variation in the susceptibility of species and strains of* Trichophyton *antifungal to agents*

Isolates	Griseofulvin		Miconazole	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
T. mentagrophytes				
HM 331, 385	1.5	2.5	0.5	1.5
HM 325	1.5	2.5	2.5	5.0
HM 147	2.5	2.5	1.5	1.5
HM 106	5.0	3.0	1.5	5.0
T. rubrum				
HM 95, 183, 24, 280, 386	0.5	1.5	0.1	1.5
T. violaceum				
HM 72	0.1	1.5	0.1	1.5
HM 163, 299, 377, 364	0.5	1.5	0.1	1.5

MIC—Minimum Inhibitory Concentration.
MFC—Minimum Fungicidal Concentration.

centration range of 1.5 to 5.0 μg/ml. Miconazole was more effective and showed fungicidal effect at a concentration of 1.5-2.5 μg/ml. Strains of *T. violaceum* and *T. rubrum* were susceptible to both the drugs (Table 7). Strains of *T. mentagrophytes* exhibited slight variation. Griseofulvin was found to inhibit the growth of 4 strains at 1.5 to 2.5μg/ml level and was fungicidal at 2.5 μg/ml. The strain HM 106 was resistant to griseofulvin. It required two-fold higher concentration to cause inhibition and twelve-fold higher concentration for fungicidal action.

Susceptibility of *Candida* species to various antifungal agents is shown in Table 8.

Table 8. *Susceptibility of* Candida *species and strains to antifungal agents*

Species and No. of isolates	Buclosamide		Nystatin		Miconazole	
	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)	MIC (μg/ml)	MFC (μg/ml)
C. albicans (14)	6.25-25 (4)*	25 (7)**	1.56-12.5	1.56-25	1.56-3.12	1.56-3.12
C. pseudo tropicalis (12)	6.25-12.5	25 (1)**	1.56-6.25	3.12-12.5	1.56-6.25	1.56-6.25
C. krusei (3)	6.25-12.5 (1)*	(3)**	3.12-6.25	3.12-12.5	1.56-3.12	1.56-3.12
C. parakrusei (2)	6.25-25	25	12.5-25	12.5-(1)**	3.12	3.12
C. parapsilosis	(1)*	(1)*	6.25	6.25	1.56	1.56
C. tropicalis	3.12	6.25	1.56	3.12	1.56	3.12

MIC—Minimum Inhibitory Concentration.
MFC—Minimum Fungicidal Concentration.
*not inhibited even at 25 μg/ml.
*not fungicidal even at a concentration of 25 μg/ml.

Miconazole inhibited the growth and affected the viability of all the species and strains of *Candida* at a concentration range of 1.5 to 6.25 μg/ml. While majority of yeasts were inhibited by the nystain at 1.56 to 12.5 μg/ml, a few required a concentration of 25 μg/ml. Buclosamide inhibited yeasts at higher concentrations, viz., 6.25 to 25 μg/ml. It showed fungicidal effect only in 50% of *C. albicans* strains at 25 μg/ml.

DISCUSSION

The widespread occurrence of dermatophytes in the tropics is well known and the present study indicates that they are very common in this part of the country affecting the people of all classes.

A fairly significant percentage of people had tinea versicolor, with microscopically demonstrable ovoid spore masses and short hyphae in scales. Our attempts to cultivate the causal organism either by using Martin-Scotts medium[11] or by following the method of Shing Lin Lii[10], were not successful.

In the present study *T. rubrum* is found to be the common etiological agent, and this finding is in conformity with earlier reports from our laboratory and other parts of India. The second common etiological agent encountered was *E. floccosum*. A similar high incidence of *E. floccosum* was reported from Bengal by Ghosh[3]; while *T. mentagrophytes* was reported to be the second common etiological agent in other parts of the country. *M. canis* was isolated from only one case for the first time in this part of the country.

Another salient feature of the present study is significantly high incidence of yeasts in superficial mycotic cases. Their incidence, isolation, characterisation[15] and their pathogenic ability[16] have been studied in greater detial and reported elsewhere.

The predominance of infections in men may be attributed in general to excessive sweating and abrasion of under garments, particularly in case of tinea cruris. Both tinea corporis and tinea cruris were observed in great numbers in factory workers than in any other group. Women, house-wives in particular, were mostly affected by tinea corporis infections, on foot, nail and intertrigal regions. In women, the tinea corporis was observed chiefly around the waist aud under arms because of the type of clothing they wear. The tightly worn sarees and blouses cause abrasions in the skin, and sweat provides a mircroclimate suitable for the maintaince of the invading fungus. Act on and McGuire[1] have described the predisposing conditions for tinea, as thinness of the skin surface, moisture as in obese people friction by clothing and shoes, and constant wetting of hands and feet. Incidence of scalp infections only among children up to the age of 18 years and below is an interesting observation in conformity with earlier reports.

The dermatophytes isolated from these clinical materials have been tested for their pathogenicity in experimental animals like mice, rates and guinca pigs and some of them were found to be pathogenic (detail's of experimental infection and pathogenicity of dermatophytes to be published elsewhere).

The *in vitro* susceptibility of dermatophytes and yeasts to various antifungal agents was examined. Miconazole appeared to be highly potent than other drugs tested. Dermatophytes were more susceptible than yeasts. Griseofulvin and nystatin were effective at two-fold higher concentrations. Buclosamide needed higher concentrations for its fungi static and fungicidal action both on dermatophytes and yeasts. The clinical efficacy of miconazole as a topical agent has been studied and reported by us recently,[17] showing 100% cure rate in cases of dermatophytes and yeasts without any recurrence after the therapy is discontinued.

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15

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Yeasts in Superficial Mycosis I. Incidence, Isolation and Characterisation

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In a recent study extended over a preiod of 15 months, 320 patients attending the skin department of a local hospital, have been examined for the etiological agents in skin infections. In nearly 31 per cent of the cases which are positive by culture, yeasts are isolated as sole causative organisms. Mixed infections of yeasts with dermatophytes are observed in another 9 per cent and the associated dermatcphytes are **E. floccosum** and **T. rubrum**. Yeasts are isolated not only from hands, feet and nalls, but also frcm body and groin. In all 42 yeast-like organisms are isolated frcm 37 patients with different clinical types. Based on their morphology in different media, fermentation tests, pseudomycelia, chlamydospore and germ-tube formation, 33 isolates are characterised as to their species. They included **C. albicans, C. pseudotropicalis, C. krusei, C. parakrusei, C. parapsilosis, C. guillermondii** and **C. tropicalis**. In this study **C. albicans**, a definitely proved pathogen accounted for 33 per cent of the yeasts islcated. Further, a high incidence of **C. pseudotropicalis**, (28 per cent) is also observed.

Introduction

Among fungi pathogenic to humans and animals, importance of yeasts is well recognized. Most of the pathogenic yeasts exist as commensals in humans and animals and are frequently isolated from mouth, intestine, vagina **etc.** However, during the altered physiological conditions of the host as in infancy, pregnancy, hormonal disorders, diabetes and due to prolonged treatment with broad spectrum antibiotics, costicosteroids **etc.**, yeast-like organisms could be invasive and bring about pathogenic manifestations (Chakravarthi **et al.**, 1962; Balbir Singh and Sharma, 1962; Daftary **et al.**, 1962 and Raman **et al.**, 1962). In surveys of the incidence of fungal diseases of both systematic and superficial nature, yeasts are found to be prominent etiological agents (Ghosh, 1948; Desai **et al.**, 1962; Indira **et al.**, 1971).

In the present study from June 1972 to August, 1973, 320 patients with suspected fungal infections attending the skin department of Employee's State Insurance Hospital, Rajajinagar, Bangalore have been examined. Besides various species of dermatophytes, yeast-like organisms have been isolated from superficial skin lesions and characterised to their species.

Material and Methods

Clinical material : Scrapings are collected from patients by means of sterile scalpel after clearing the site with 70 per cent ethyl alcohol.

Direct examination : Scrapings are digested with 10 per cent KOH and then microscopically examined for the presence or absence of fungal elements.

Culture : Sabouraud's glucose (S.g.) agar supplemented with chloramphenicol (0·04 mg./ml.) and cycloheximide (0·5 mg./ml.) and Littman Oxgall agar, are used for the primary isolation. Scrapings are placed on agar slants and incubated at room temperature. When yeast-like colonies appeared, they are transferred on to S.g. agar slants, allowed to grow for 2 days at 30°C. and then stored at 0-5°C.

Morphology of growth in S.g. broth : Isolates are inoculated into S.g. broth, incubated at 30°C. and then observed for the formation of surface growth or submerged growth.

Fermentation of sugars : The ability of all the isolates to ferment the sugars glucose, sucrose, maltose and lactose is tested according to the method of Beneke and Rogers, 1971. Tubes contained 4·5 ml. of sterile beef extract broth and 0·5 ml. of 10 per cent sterile aqueous sugar solution. The broth contained bromocersol purple as pH indicator (0·16 mg./ml.) and Durham tubes to collect the gas evolved. These tubes are inoculated with 0·1 ml. of 18 h. culture of the isolates in S.g. broth, then incubated at 37°C. for 48 h. and observed for the production of acid and gas. The fermentation properties are found to be same even after 72 h.

Production of pseudomycelia and chlamydospores : The relative efficiency of cornmeal agar, chlamydospore agar (Moss and McQuoun, 1969) and rice agar with Tween 80 (Beneke and Rogers, 1971) to facilitate pseudomycelia and chlamydospore formation is examined.

Rice agar with Tween 80 is found to yield best results when tested with a standard strain of **C. albicans** (Z 248) within 20 h. incubation. The chlamydospore production was maximum at 20°C. than at 30 or 37°C. Hence rice agar with Tween 80 medium and incubation at 20°C. are chosen for screening all the isolates for their ability to produce pseudomycelia and chlamydospores.

Formation of germ tubes in the presence of serum and egg albumin : Rabbit and guinea pig sera are used to test the formation of germ tubes (Beneke and Rogers, 1971) with the standard strain. Serum samples are inoculated and incubated at 37°C. After 4 h. of incubation, small delicate germ tubes are found on microscopic examination with a standard strain of **C. albicans**, Z248. Hen's egg albumin (Gentles and La Touche, 1969) is found to be equally suitable and it facilitated the production of more pronounced and stout germ tubes. Hence the hen's egg albumin has been used for screening all the isolates.

Standard strains tested : In all the above experiments, parallel tests are performed on standard strains of **Candida** species, **viz., C. albicans** Z248, **C. Krusei** Z70, **C. pseudotropicalis** Z27 (got from London School of Hygiene and Tropical Medicine,

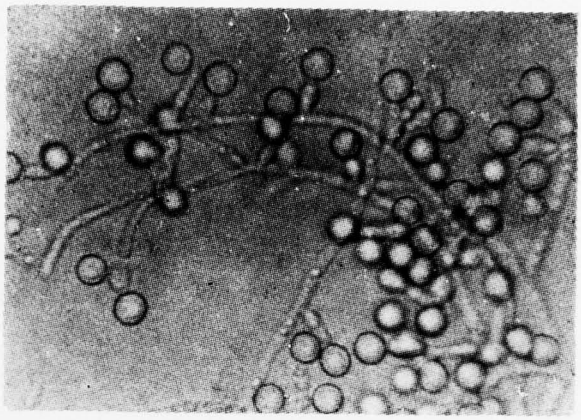

Fig. 1. Chlamydospore production in C. albicans. HM312, ×480.

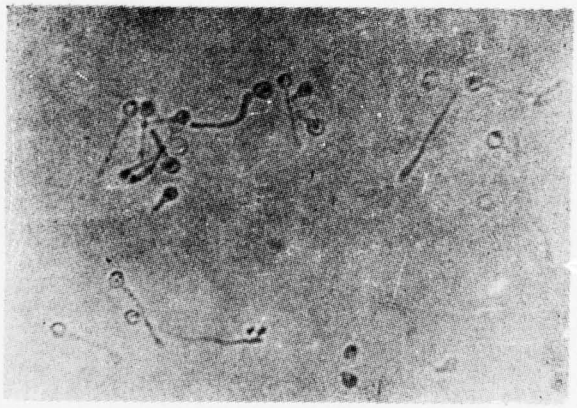

Fig. 2. Germ tube production in C. albicans. HM312, ×480.

London), and **C. tropicalis** 509/7 (got from Vallabhbhai Patel Chest Institute, Delhi University, Delhi) to serve as controls.

Results and Discussion

The results of the study of 320 cases of suspected superficial mycotic infections are shown in Table I. In all, 93 cases (29·0 per cent) yielded causal organisms upon culture and they included both dermatophytes and yeasts. Dermatophytes accounted for 56 cases (out of 93) and the isolated species are : **T. mentagrophytes** (8), **T. rubrum** (18), **T. violaceum** (10), **E. floccosum** (20).

Table I. Yeast-like organisms in superficial mycoses*.

Site of infection	No. of cases	Causal organisms encountered				
		T. mentagrophytes	T. rubrum	T. violaceum	E. floccosum	Yeast
Scalp	3	..	..	3	..	..
Beard	1	..	..	1	..	..
Body	37	5	10	6	7	9†
Groin	35	3	8	.	12	12‡
Hand (interdigital region)	5	..	..	..	1	4
Foot	6	..	..	..	..	6
Nail and associated skin	6	..	..	..	..	9
Total	93	8	18	10	20	37

*In 3 cases yeasts were isolated along with **E. floccosum** (2 cases) and **T. rubrum** (1 case).
†In 5 cases yeasts were isolated along with **E. floccosum** (3 cases) and **T. rubrum** (2 cases).
‡The data is from 320 cases studied.

Among 93 cases that are positive by culture, yeast-like organisms accounted for 37 cases (40 per cent). They are isolated not only from hands, feet, nails and associated skin regions but also from body and groin. While dermatophytes are predominant etiological agents causing infections of body and groin (51 cases), yeasts are also responsible in significant number (21) of cases (29 per cent).

In all, 42 yeast-like organisms are isolated from 37 patients (in 5 patients 2 yeast-like organisms are isolated from each) with different clinical types. Based on their morphology in S.g. broth, rice agar, fermentation tests etc., 33 isolates are characterized, as species of **Candida**, while the remaining 9 isolates are not identified because of the varied fermentation reactions and lack of pseudomycelia formation. The following 7 species of **Candida** are encountered (Table II).

Group 1 : **C. albicans** (14 isolates) HM12, 27, 41a, 42, 66, 144, 206, 281b, 303, 309, 312, 318a, 318b, 343.

Group 2 : **C. pseudotropicalis** (11 isolates) HM40, 82a, 116b, 244, 282, 290b, 295, 322a, 322c, 323b, R^1.

Table II. Characterisation and identification of yeast-like organisms.

Organisms (isolated HM strains)	Fermentation behaviour				Growth in S.g. broth	Morphology in rice agar	Production of germ tube	Species identified
	Glu	Mal	Suc	Lac				
Group 1 (14 isolates)	AG	AG	A	..	No surface growth	Pseudomycelia bearing terminal chalmydospores	+	**C. albicans**
Group 2 (11 isolates)	AG	—	—	AG	No surface growth	Pseudomycelia	—	**C. pseudo-tropicalis**
Group 3 (3 isolates)	AG	—	—	—	Frothy mycelial growth	Pseudomycelia	—	**C. krusei**
Group 4 (2 isolates)	AG	—	—	—	No surface growth	Pseudomycelia	—	**C. parakruse**
Group 5 (1 isolate)	AG	AG	A	—	No surface growth	Pseudomycelia	—	**C. parapsi-lopsis**
Group 6 (1 isolate)	AG	—	A	—	No surface growth	Pseudomycelia	—	**C guillier-mondii**
Group 7 (1 isolate)	AG	A	AG	—	Surface growth	Pseudomycelia	—	**C. tropicalis**
Group 8 (9 isolates)	Varied reactions				No surface growth	Blastospores	—	Unidentified

Group 3 : C. **krusei** (3 isolates) HM41b, 308b, R^3.

Group 4 : C. **parakrusei** (2 isolates) HM265, 123.

Group 5 : C. **parapsilosis** (1 isolate) HM152.

Group 6 : C. **guilliermondii** (1 ioslate) HM240.

Group 7 : C. **tropicalis** (1 isolate) HM279.

Group 8 : Unidentified (9 isolates) HM74, 155, 204, 252, 280, 281a, 288, 289, 310.

The identity of isolates in group 1 as **C. albicans** is further confirmed by their ability to produce chlamydospores (Plate XXIII, Fig. 1) and germ tubes in presence of egg albumin (Plate XXIII, Fig. 2).

Several species of yeasts belonging to the genus **Candida** are isolated from superficial mycoses. In this study, **C. albicans**, a definitely proved pathogen, accounted for nearly 33 per cent of the yeasts isolated; further a high incidence of **C. pseudotropicalis**, i.e., 11 out of 42 (26 per cent) is of interest.

The present study establishes the occurrence of yeasts in dermatological lesions. Whether they are present merely as saprophytes or responsible for the dermatological lesions needs further study on their pathogenic ability in experimental animals.

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20

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Yeasts in Superficial Mycosis : Pathogenicity of Candida Species to Swiss Albino Mice

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Pathogenicity of several species and strains of *Candida* isolated from superficial skin infections, in mice has been examined. Fifty per cent of yeasts isolated were found to be pathogenic. In addition to *C. albicans* which is a definitely known pathogen, other species of *Candida viz., C. pseudotropicalis* and *C. parapsilosis* have been found to be equally pathogenic.

Introduction

Although the occurrence of yeasts, notably species of *Candida*, as commensals in various parts of the human body, *viz.*, mouth (Young *et al.*, 1951), intestine (Marples and di Menna, 1952) and vagina (Balbir Singh and Sharma, 1962) and their pathogenicity under altered physiological conditions, *viz.*, infancy (Taschdjian and Kozinn, 1957), old age, pregnancy (Daftary *et al.*, 1962), prolonged hormone and broad-spectrum antibiotic treatment (Crossby, 1967 and Raman *et al.*, 1962) are well documented, their occurrence and function in superficial skin layers are less understood. While Drouhet (1960) reported failure to recover *C. albicans* from a large number of healthy skin samples, its definite presence and relation to age have been shown by Marples and Somerville (1966). However, information is lacking on species of *Candida* which are frequently encountered on the skin and their potential pathogenicity. Though in earlier studies on superficial skin infections, yeast-like organisms have been isolated as etiological agents, their identity and pathogenicity are not reported.

The incidence, isolation and characterisation of yeast-like organisms from superficial skin infections have been reported earlier by the present authors (Vijaya Manohar *et al.*, 1975). The present paper deals with the pathogenicity of these yeast-like organisms to Swiss albino mice.

Material and Methods

Organisms : The following strains of *Candida* species (isolated from patients designated as HM series) and strains obtained from London School of Hygiene and Tropical Medicine, London (designated as Z series) were used.

C. albicans : HM12, 27, 41a, 66, 206, 303, 309, 312, 318a, 318b and Z248.

C. pseudotropicalis : HM40, 56, 82a, 116b, 244, 282, 290b, 295, 322a, 322b and Z27.

C. parakrusei : HM265.

C. parapsilosis : HM152 and Z40.

C. tropicalis : HM279 and Z156.

Preparation of inocula : Cells from an 18 hour culture, grown at 30°C. in Sabouraud's glucose (Sg) broth, were harvested, washed twice with saline and suspended in the same. The cell population was quantitated by haemocytometer.

Experimental animals and mode of inoculation : Swiss albino mice of either sex in the weight range of 15 to 25 g. were used. Animals were infected by intravenous route following the procedure described by Ramananda Rao and Sirsi (1962). As the strain Z248, proved to be pathogenic at a dose of 5×10^6 cells with an average survival period of less than 7 days, all the isolates were tested by injecting 0·1 ml. cell suspension containing 5 and 10×10^6 cells. Isolates which were non-pathogenic at a dose level of 10×10^6 cells were also tested at a dose level of 30×10^6 cells. Each group consisted of 5 animals and control group received 0·1 ml. of saline. Body weights of the experimental animals were recorded before and during the course of infection at regular intervals.

Examination of the tissue preparation : The mice were kept under observation and mortality rates were calculated at the end of 30 days. The mice died of infection and those which survived, were sacrificed at the end of one month, dissected and examined for the presence of macroscopic lesions in organs like lung, liver, spleen, kidney and brain. Smears prepared from these tissues were microscopically examined (after staining with 1 per cent aqueous methylene blue) for the presence or absence of blastospores and pseudomycelia.

Culturing of the organisms : S.g. agar and broth containing cycloheximide (0·5 mg./ml.) and chloramphenicol (0·04 mg./ml.) were inoculated with aliquots of tissue homogenates and incubated at 30°C.

Results and Discussion

The animals infected with pathogenic strains showed loss in weight. Pathogenicity of strains of *C. albicans* is shown in Table I. The strain Z248 was found pathogenic at both the dose levels of 2·5 and $5·0\times10^6$ cells, with average survival periods of 10·5 and 6·2 days, respectively. All the isolates of *C. albicans* tested were found pathogenic to varying degrees. Four of them (40 per cent) were highly pathogenic with an average survival time of 1·2 to 3·2 days and the rest were moderately pathogenic with an average survival period of 6·8 to 10·5 days.

Among 10 isolates of *C. pseudotropicalis*, 4 strains were found to be pathogenic to varying extent as seen in strains of *C. albicans*. Isolates HM295 and 322a were highly pathogenic (Table II). With a two-fold increase in the dose of inoculum, the average survival time of the animals infected with these isolates was considerably reduced

Fig. 1

Lesions in kidneys of mouse infected with *C. pseudotropicalis* HM295.

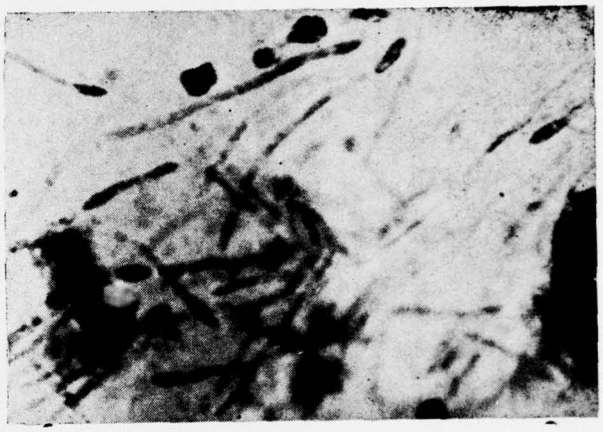

Fig. 2

Mycelia in the smears of kidneys of mouse infected with *C. pseudotropicalis* HM295. × 480.

Fig. 3

Mycelia in the smears of kidneys of mouse infected with *C. albicans* HM27. × 480.

Table I. Pathogenicity of C. albicans strains* to Swiss albino mice.

Isolates	Dose of infection	No. of animals died/No. of animals used	Average survival period (days)
Standard strain			
Z248	$2{\cdot}5 \times 10^6$ cells	4/5	10·5
,,	$5{\cdot}0 \times 10^6$,,	4/5	6·2
Highly pathogenic			
HM303	$5{\cdot}0 \times 10^6$ cells	4/5	1·2
HM12	,,	3/5	2·3
HM312	,,	4/5	2·7
HM41a	,,	5/5	3·2
Moderately pathogenic			
HM66	,,	5/5	6·8
HM206	,,	3/5	8·1
HM318b	,,	5/5	8·6
HM27	,,	5/5	8·6
HM318a	,,	2/5	9·0
HM309	,,	4/5	10·5

*Since all the strains were found to be pathogenic at a dose of 5×10^6 cells, higher doses were not tested.

Table II. Pathogenicity of C. pseudotropicalis strains to Swiss albino mice.

Isolates	Dose of infection	No. of animals died/No. of animals used	Average survival period (days)
Pathogenic*			
HM295	5×10^6 cells	5/5	5·0
,,	10×10^6 ,,	5/5	2·8
HM322a	5×10^6 ,,	3/5	8·3
,,	10×10^6 ,,	4/5	3·5
HM56	5×10^6 ,,	0/5	...
,,	10×10^6 ,,	4/5	5·0
HM82a	5×10^6 ,,	0/5	...
,,	10×10^6 ,,	3/5	8·0
Non-pathogenic			
HM40, 116b, 244,	5×10^6 ,,	0/5	..
290b, 322c,	10×10^6 ,,	0/5	..
322b and Z27	30×10^6 ,,	0/5	..

*Strains which proved pathogenic at a dose level of 10×10^6 cells, were not tested at a dose level of 30×10^6 cells.

(5·0 to 2·8 days and 8·3 to 3·5 days). The other two isolates *viz.*, HM56 and 82a were moderately pathogenic at a dose of 10×10^6 cells, with an average survival period of 5·0 and 8·0 days, respectively. The remaining isolates and the strain Z27 were non-pathogenic even at a dose of 30×10^6 cells.

Among other species tested, *C. parapsilosis* HM152 was moderately pathogenic at a dose of 10×10^6 cells (Table III). The tissue involvement in animals infected with pathogenic strains is shown in Table IV. Only few strains *viz.*, *C. albicans* HM27, *C. pseudotropicalis* HM295 (Plate CIV, Fig. 1), HM322a and *C. parapsilosis* HM152 produced discrete lesions in kidneys. However kidney smears from all the animals infected with pathogenic strains showed yeasts and pseudomycelia on microscopic examination (Plate CIV, Figs. 2 and 3). No such macroscopic lesions were seen in other organs *viz.*, lung, liver, brain and spleen, and microscopically smears did not show any yeasts or pseudomycelia. But homogenates from all the organs *viz.*, kidney, spleen, liver, lung and brain, yielded yeasts on culture. Recently similar findings have been reported in case of *C. albicans* by Marianita and Schmitt (1973). No macroscopic lesions were found in any of the organs of the animals which survived and were sacrificed at the end of one month, and tissue smears from these organs did not reveal either blastospores or pseudomycelia. Further, homogenates from any of the organs from these animals, did not yield yeasts on culture.

In the present study, the pathogenic ability of 50 per cent of the yeasts isolated from skin lesions is established. In addition to *C. albicans* which is a definitely known pathogen, other species of *Candida*, *viz.*, *C. pseudotropicalis* and *C. parapsilosis* are found to be pathogenic. Since these organisms were isolated from superficial mycotic lesions (Vijaya Manohar *et al.*, 1975) and found to be pathogenic in experimental animals, the

Table III. Pathogenicity of Candida spp. to Swiss albino mice.

Isolates	Dose of infection	No. of animals died/No. of animals used	Average survival period (days)
C. parapsilosis HM152*	5×10^6 cells	0/5	–
,,	10×10^6 ,,	4/5	13
C. parapsilosis Z40	5×10^6 ,,	0/5	..
C. tropicalis HM279, Z156	10×10^6 ,,	0/5	...
C. parakrusei HM265	30×10^6 ,,	0/5	..

*Since this strain proved to be pathogenic at a dose level of 10×10^6 cells, it was not tested at 30×10^6 cells.

Table IV. Tissue involvement in experimental candidiasis in Swiss albino mice.

Isolates	Species	Macroscopic lesions in organs	Microscopic examination of kidney smears*
Highly pathogenic			
HM303	C. albicans	No lesions in kidneys and other organs	Yeasts and pseudomycelia
HM12	,,	,,	,,
HM41a	,,	,,	,,
HM312	,,	,,	,,
HM295	C. pseudotropicalis	Discrete lesions in kidneys	,,
HM56	,,	No lesions in kidneys and other organs	,,
HM322a	,,	Discrete lesions in kidneys	,,
Moderately pathogenic			
HM66	C. albicans	No lesions in kidneys and other organs	,,
HM82a	C. pseudotropicalis	,,	,,
HM206	C. albicans	,,	,,
HM318a	,,	,,	,,
HM27	,,	Discrete lesions in kidneys	,,
HM309	,,	No lesions in kidneys and other organs	,,
HM152	C. parapsilosis	Discrete lesions in kidneys	,,

*Smears from lung, liver, brain and spleen did not reveal any yeast-like organisms or pseudomycelia on microscopy but the homogenates from all these organs yielded yeasts upon culture.

need for cultural diagnosis is emphasized in selecting a suitable therapy for superficial mycotic infections.

Acknowledgment

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C.2. ANTIMYCOTIC DRUGS

Since the etiology of superficial mycotic infections are highly variable, the study of their *in vitro* susceptibility to antimycotic drugs would help in selecting a suitable therapeutic agent and its dosage. Though a wide spectrum of antimycotic agents have been reported, the number of drugs used in practice are very few and often are highly selective in their action on fungi. There is no single drug which acts on all the etiological agents with equal efficacy. The *in vitro* susceptibility of various superficial mycotic agents to some of the commonly used antifungal agents like griseofulvin, nystatin and buclosamide has been examined. Their antifungal activity was compared with miconazole nitrate (a synthetic antifungal drug with broad-spectrum action). The results revealed that both dermatophytes and *Candida* spp. were highly susceptible to miconazole as compared to griseofulvin or nystatin or buclosamide. Further the clinical evaluation of miconazole as a topical applicant in patients with superficial mycotic infections has shown that the drug effectively cures all the superficial mycotic infections irrespective of the etiological agents involved (pages 11, 12,27-30).

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Mycopathologia vol. 59, 1, pp. 57–60, 1976

MICONAZOLE IN THE TREATMENT OF SUPERFICIAL MYCOSES*

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Abstract

Miconazole nitrate (2%) cream was evaluated in the treatment of superficial mycoses. Out of 116 patients having multiple clinical diagnoses, 66 cases were found to be positive by culture. Species of *Trichophyton* were the predominant etiological agents (in over 60%) followed by *Candida* species (20%) and *Epidermophyton floccosum* (15%). All the cases selected for study were followed up to a period of 4-18 months. A cure rate of 94.6 per cent was observed in all the cases where causal organisms were isolated. Significantly high cure rate (66%) was also seen in cases where causal organisms could not be isolated, including cases of tinea versicolor. Results of mycological examination were in confirmity with the clinical results.

Introduction

Miconazole nitrate has been shown to have a broad-spectrum of in vitro antimicrobial activity. It inhibits the growth of dermatophytes, pathogenic and nonpathogenic yeasts and gram-positive bacteria (2, 4, 11, 15). Its effective therapeutic use as a topical applicant in treating skin and nail infections and in vaginal candidiasis has been recognised (1, 3, 5, 10, 12, 13, 14). Its use under tropical conditions in India necessitated a clinical and mycological evaluation in a random population of patients.

* Read at the Symposium on Human Mycoses and Miconazole held in New Delhi, India, February, 1975.

Patients and methods

Cases were selected from those attending a local hospital, who consented to take the treatment and to report periodically for examination. Irrespective of their age, sex, severity and duration of infection, with or without the history of previous treatment with available antifungal agents, each patient was supplied with miconazole nitrate in the form of 2% cream in collapsible tubes. Each patient was asked to smear the lesions with the cream at least twice a day. During the night the drugsmeared areas were covered with butterpaper (occlusive method) and bandaged. The extent and severity of the lesions were recorded and photographs of few cases were taken before and after cure. Patients were examined once in a week and at each attendance the skin and nail scrapings were collected by the dermatologists and examined by microscopy and culture in the laboratory.

Several representative pieces of skin and nail scrapings were examined microscopically for the presence of fungal elements after digesting with 10 per cent potassium hydroxide. Sabouraud's glucose agar supplemented with chloramphenicol (0.04%) and cycloheximide (0.5%) was inoculated with skin and nail scrapings and incubated at 30°C for three weeks, to isolate dermatophytes and yeasts.

Miconazole treatment was continued for 30 days after the clinical cure and later discontinued. At each attendance patients were questioned as to adverse reactions to the preparation they were using and whether they found the preparation acceptable. An attempt was made to follow-up all the patients for a period of 4-18 months.

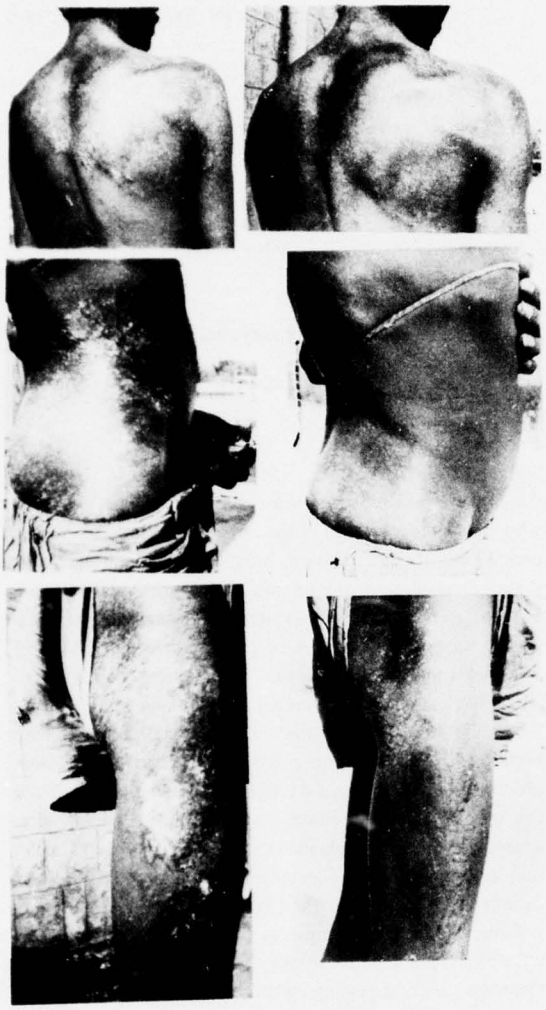

Fig. 1. Efficacy of miconazole in tinea corporis infections.

Table 1. Superficial mycotic cases taken for miconazole treatment

	No. of patients	No. of clinical diagnoses encountered*
Total number of cases selected for miconazole treatment	116	141
Number of cases positive by culture	66	80
Number of cases not positive either by microscopy or by culture	50	61

* Some patients had more than one type of clinical lesions

Table 2. Efficacy of miconazole treatment* in superficial mycoses caused by dermatophytes and yeasts

Clinical diagnoses	No. of cases encountered	Organisms isolated (Nos.)	Cured	Not cured	Not traced
tinea cruris	29	*T. rubrum* (12) *E. floccosum* (8) *Candida* spp. (4) *T. mentagrophytes* (4) *T. violaceum* (1)	29	-	-
tinea circinata	19	*T. rubrum* (12) *E. floccosum* (2) *Candida* spp. (1) *T. mentagrophytes* (2) *T. violaceum* (1) *M. canis* (1)	16	-	3
tinea corporis	11	*T. rubrum* (7) *T. mentagrophytes* (4)	11	-	-
tinea pedis	5	*T. rubrum* (1) *E. floccosum* (1) *T. violaceum* (1) *Candida* spp. (2)	4	1	-
tinea manum	1	*E. floccosum* (1)	-	1	-
intertrigo	4	*E. floccosum* (1) *Candida* spp. (3)	3	-	1
onychomycosis	6	*Candida* spp. (4) *T. violaceum* (1) *T. rubrum* (1)	3	2	1
tinea axilla	1	*Candida* spp. (1)	1	-	-
kerion	2	*T. mentagrophytes* (1) *T. violaceum* (1)	2	-	-
tinea capitis	1	*Candida* spp. (1)	1	-	-
tinea barbae	1	*T. violaceum* (1)	1	-	-
Total	80		71	4	5

*Follow-up period was 4-18 months. Drug effect: Cured 71/75 = 94.6%. Not cured 5/75 = 7%

Results

One hundred and sixteen patients with multiple diagnoses were selected for the study. Eighty clinical diagnoses from 66 patients yielded fungi upon culture (Table 1). A significantly high percentage of incidence was observed in the age group of 16-45 years who were young and healthy. The percentage distribution of these cases according to the sex was as follows – males 75, females 21 and children 3.

The results on the efficacy of miconazole treatment in cases which were positive by culture are shown in Table 2.

Out of 80 clinical diagnoses where organisms were isolated, tinea cruris accounted for 29, followed by tinea circinate in 19 and tinea corporis in 11 cases. Onychomycoses and tinea pedis together accounted for 11 cases. Though tinea cruris infections were caused by a variety of etiological agents like species of *Trichophyton*, *Epidermophyton floccosum* and *Candida* spp., all the cases were completely cured by miconazole. Similarly over 90 per cent cure rate was seen in tinea circinata and tinea corporis infections. In tinea pedis

Table 3. Efficacy of miconazole in the treatment* of superficial mycotic cases where etiological agent was not isolated

Clinical diagnoses	Total	Cured	Not cured	Not traced
tinea cruris	14	12	2	-
tinea circinata	10	7	2	1
tinea corporis	6	5	-	1
tinea pedis	4	1	3	-
tinea manum	6	-	5	1
tinea intertrigo	4	1	-	3
onychomycosis	6	2	3	1
tinea axilla	2	1	1	-
tinea capitis	1	1	-	-
tinea versicolor	8	6	2	-
Total	61	36	18	7

*Follow-up period was 4-18 months

and onychomycoses where *Candida* spp. were mostly the causal organisms, miconazole treatment showed complete cure in 70 per cent of the cases. When all the 75 clinical diagnoses were taken into account, the cure rate was 94.6 per cent.

A high cure rate (66%) was noticed even in cases where causal organisms could not be isolated (Table 3).

Comments

Species of *Trichophyton* were encountered in majority of cases, *T. rubrum* being the most dominant etiological agent, causing the infections in groin and the corporial regions of the body. Topically applied miconazole was found to be highly effective in the treatment of infections caused by both dermatophytes and yeasts as shown by clinical and mycological examinations during the treatment and follow-up. There were no reports of relapse after the discontinuation of the miconazole treatment during the follow-up period ranged from 4-18 months or of adverse side effects with the use of miconazole cream. These findings are in conformity with the prior reports of the efficacy of the miconazole in curing superficial mycoses.

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C.3. MOLECULAR BASIS OF ACTION OF MICONAZOLE

Miconazole nitrate [1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate], a recently discovered synthetic imidazole derivative, is a potent antifungal drug. The drug is clinically used in the treatment of superficial mycoses (including those of Candida species) and systemic mycoses.

Though the chemotherapeutic usefulness of miconazole has been well documented, the mechanism by which it inhibits the growth of susceptible organisms is not known. Hence, investigations have been carried out to elucidate its molecular basis of action. The main objectives are to relate the biological effects of the drug on sensitive cells to the interaction between the drug and its biochemical target in the cells and ultimately to explain these interactions in molecular terms. Secondly, these studies would explain the basis of selective toxicity of the drug. A detailed understanding of the antifungal action of the drug at the molecular level may generate new ideas for the design of entirely novel drugs. Further, the drug can be used as a probe to understand in detail the biochemical processes which it inhibits.

With the above objectives in mind investigations have been made using three different biological systems. These are: (a) cells of Candida albicans, a pathogenic yeast, (b) mammalian erythrocytes, and (c) rat liver lysosomes. Miconazole inhibits the growth of C. albicans by impairing the cell membrane function resulting in the loss of intracellular materials (pages 34-39). A close correlation is observed between the loss of intracellular materials and loss of viability of cells. The uptake of labeled precursors and their incorporation into DNA, RNA, protein

and lipid are inhibited by miconazole as a result of cell membrane damage caused by the drug. Further, the drug brings about hemolysis of mammalian erythrocytes by direct interaction with the erythrocyte membrane (pages 40-45). Miconazole binds strongly to membrane lipoprotein fractions. Miconazole-induced hemolysis is inhibited by serum and the serum components responsible for the inhibition have been identified as IgG and albumin. Studies have been extended further to examine the effect of miconazole on subcellular membranes using rat liver lysosomes as a model system. Miconazole exerts a profound effect on the lysosomal membrane and causes release of lysosomal enzymes (pages 46-50). The results obtained so far clearly reveal that the drug interacts with both cellular and subcellular systems and impairs their membrane structure and function. The interaction of miconazole with the membrane constituents present in these divergent biological systems brings about disorganization of the structure of membranes to an extent that they lose their properties as permeability barriers.

A major achievement in these studies is that the biochemical target for the drug has been identified as membrane in both cellular and subcellular systems. The present approach to study the molecular basis of action of miconazole using three different biological systems has yielded a more comprehensive view of biological action of the drug. These studies further show that the drug interacts with divergent biological systems such as yeasts, erythrocytes and lysosomes. Further studies on the characterization of the binding site of the drug on the membrane and also its inter-

action with model membranes will provide a greater insight into the understanding of the action of miconazole and would throw more light on the subject of membrane biochemistry itself. These studies are in progress.

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Studies on the Mechanism of Action of Miconazole: Effect of Miconazole on Respiration and Cell Permeability of *Candida albicans*

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The antifungal drug, miconazole nitrate, inhibits the growth of several species of *Candida*. *Candida albicans*, one of the pathogenic species, was totally inhibited at a concentration of approximately 10 μg/ml. Endogenous respiration was unaffected by the drug at a concentration as high as 100 μg/ml, whereas exogenous respiration was markedly sensitive and inhibited to an extent of 85%. The permeability of the cell membrane was changed as evidenced by the leakage of 260-nm absorbing materials, amino acids, proteins, and inorganic cations. The results we present clearly show that the drug alters the cellular permeability, and thus the exogenous respiration becomes sensitive to the drug.

Miconazole nitrate [1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate] is a potent antifungal drug (4), the structure of which is shown in Fig. 1. The drug was obtained as a gift sample through the courtesy of Ethnor Ltd., Bombay, India. It is a white microcrystalline powder, has a molecular weight of 479, and is soluble in 50% ethanol on warming.

Miconazole has a broad spectrum of in vitro antimicrobial activity. It inhibits the growth of dermatophytes, namely, species of *Trichophyton*, *Microsporum*, and *Epidermophyton*, pathogenic and nonpathogenic yeasts, and gram-positive bacteria (4, 12). Its effective therapeutic use as a topical applicant in treating skin and nail infections in man and in vaginal candidiasis has been reported (2, 5, 16, 18). However, the mode of action of this drug has not yet been elucidated. In this paper we report our findings on the effect of miconazole on respiration and cell permeability of a pathogenic strain of *Candida albicans*, Z248.

MATERIALS AND METHODS

The microorganisms used in this study were obtained from the following sources: *Candida albicans* Z248, *C. parapsilosis* Z40, *C. pseudotropicalis* Z27, *C. krusei* Z70, *C. tropicalis* Z156, *C. pelliculosa* Z220, and *C. guilliermondii* Z55, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London; *C. albicans* 502/9, *C. intermedia* 512/9 and *C. tropicalis* 502/7, V.P. Chest Institute, New Delhi, India. Cultures were maintained on Sabouraud glucose agar slants.

Cell growth. For growth studies both Sabouraud glucose medium containing 2% glucose and 1% Difco neopeptone, and synthetic medium containing 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, and 30 μg of biotin per liter were used. The inoculum was prepared by growing the cells in 250-ml Erlenmeyer flasks containing 100 ml of medium for 18 h on a rotary shaker (200 rpm) at 30 C.

Growth inhibition studies were carried out in 100 ml of Sabouraud or synthetic medium in 250-ml Erlenmeyer flasks with side arms. Required volumes of miconazole (dissolved in 50% ethanol) were added aseptically to the medium to give various drug concentrations. The flasks were inoculated with 0.1 ml of an 18-h culture of the organism grown in the same medium. Control flasks contained equal volumes of 50% ethanol. They were incubated at 30 C on a rotary shaker, and the growth was measured in a Klett-Summerson colorimeter using a no. 42 filter.

Cell viability. The viability of the cells was determined by serially diluting the samples with NaCl-peptone-water (0.5% NaCl, 0.1% peptone). A portion (0.1 ml) of the diluted sample was thoroughly mixed with 2 ml of molten soft agar (1.0% peptone, 0.5% NaCl, and 0.7% agar) at 45 C and poured over the surface of Sabouraud glucose agar plates. The plates were incubated for 48 h at 30 C.

Respiration studies. Studies on respiration were carried out using standard manometric techniques (17). Cells grown in Sabouraud glucose medium for 18 h were harvested and washed three times with physiological saline and suspended in 0.05 M phosphate buffer, pH 7.0. Warburg flasks contained in a final volume of 3.2 ml: 50 μmol of phosphate buffer, pH 7.0, 50 μmol of glucose, and 1 ml of cell suspension (12 mg dry weight) in the main compartment. The center well contained 0.2 ml of 20% KOH. In the side arm of

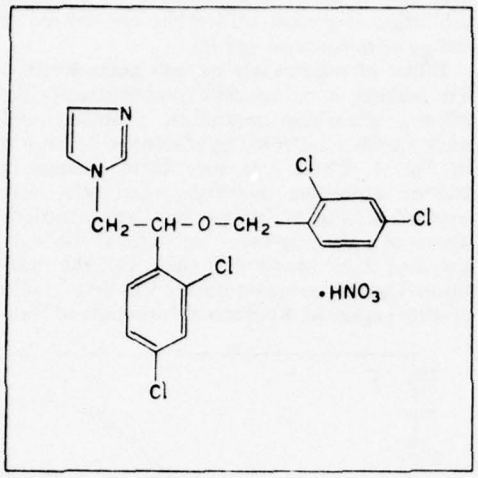

FIG. 1. *Structure of miconazole. 1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate.*

the flask, 0.5 ml of drug at appropriate concentration was taken and the control flasks contained 0.5 ml of 50% ethanol. The flasks were equilibrated for 10 min at 30 C, and the oxygen uptake was measured after the miconazole was tipped from the side arm.

Cell permeability studies. For these studies, cells were grown in Sabouraud glucose medium either in the presence or absence of [^{32}P]ortho-phosphoric acid (Bhabha Atomic Research Centre, Bombay, India) at a concentration of 0.4 μCi/ml. After 18 h of growth on a rotary shaker at 30 C, the cells were harvested and washed three times with distilled water and suspended in the same. The cells were exposed to miconazole at various concentrations on a rotary shaker at 30 C. At different time intervals samples were removed and the cell exudates were obtained by centrifugation. The cell exudates were examined for the 260-nm absorbing materials by measuring the absorbance at 260 nm, proteins were determined by the method of Lowry et al. (9), and amino acids were examined by the modified colorimetric method of Rosen (13). Potassium and sodium were estimated by flame photometry. The radioactivity in the cell exudates obtained from ^{32}P-labeled cells were determined in a Beckman LS-100 liquid scintillation counter.

RESULTS

Susceptibility of Candida species to miconazole. Various species of *Candida* have been screened for their susceptibility to the drug, and the minimum inhibitory concentration (MIC) values are shown in Table 1. Candida species markedly differed in their susceptibility to miconazole. Whereas *C. parapsilosis* and *C. pseudotropicalis* are highly susceptible, species like *C. pelliculosa, C. guilliermondii, C. intermedia,* and *C. tropicalis* needed 1,000-fold higher concentrations for their complete inhibition. In all the studies described below, a pathogenic strain of *C. albicans*, Z248, has been used. The growth patterns in Sabouraud and synthetic media and their inhibition by miconazole are shown in Fig. 2. At 10 μg/ml the growth was almost completely inhibited in Sabouraud medium, whereas at 1.0 and 0.1 μg/ml the effect was only partial. In the synthetic medium, the inhibitory effect of the drug was markedly reduced. Only 50% inhibition was noticed at 10 μg/ml.

Effect of miconazole on cell viability. The inhibition of growth of *C. albicans* in Sabouraud glucose medium was paralleled by a decline in the number of viable organisms (Table 2). The viability of cells is affected both by increasing the drug concentration and by prolonging the time of contact. At the 2.5 μg/ml level, more than 60% of cells were killed within 4 h, and at the end of 12 h of exposure the viability loss was 81%. Doubling the drug concentration had not increased the effect significantly. However, at 10 μg/ml, nearly 100% loss in viability was observed by 4 h.

Effect of miconazole on respiration. The effect of miconazole on endogenous respiration of unstarved and starved cells and exogenous respiration is shown in Fig. 3. The freshly harvested cells of *C. albicans* showed a very high level of endogenous respiration, and this level was not affected markedly by miconazole. Only at 100 μg/ml did it show a slight inhibition, and at 500 μg/ml it showed 30% inhibition. On prolonged shaking of the cell suspension for 6 h at 30 C on a rotary shaker, the endogenous respiration was markedly reduced. Even on these starved cells the drug failed to show any effect. When the endogenous respiration

TABLE 1. *Susceptibility of Candida species to miconazole*

Organism	MIC (μg/ml)[a]
Candida parapsilosis, Z40	0.01
C. pseudotropicalis, Z27	0.01
C. krusei, Z70	0.1
C. tropicalis, Z156	0.1
C. albicans, Z248	1.0
C. albicans, 502/9	1.0
C. pelliculosa, Z220	10.0
C. guilliermondii, Z55	10.0
C. intermedia, 512/9	10.0
C. tropicalis, 502/7	10.0

[a] The MIC was determined in Sabouraud glucose broth by serial tube dilution method. All the values are from duplicate determinations.

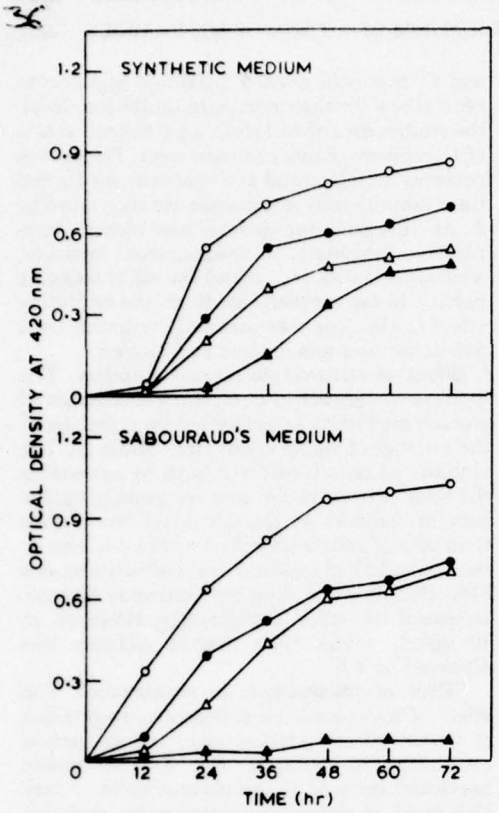

FIG. 2. *Effect of miconazole on the growth of Candida albicans. Symbols:* ○, *control;* ●, *0.1 μg/ml;* △, *1.0 μg/ml;* ▲, *10.0 μg/ml.*

TABLE 2. *Effect of miconazole on viability of Candida albicans*[a]

Conc of miconazole (μg/ml)	Loss of viability at various incubation times (%)			
	2 h	4 h	8 h	12 h
0	0	0	0	0
2.5	31	60	81	83
5.0	34	61	85	91
10.0	69	97	99	100

[a] Cells of *C. albicans* (2×10^7 cells) (18 h old) were inoculated into 100 ml of Sabouraud glucose broth in 250-ml Erlenmeyer flasks and incubated on a rotary shaker at 30 C. Samples were removed at indicated time intervals and plated as described in Materials and Methods. Colony counts were made after 48 h of incubation at 30 C.

was high, the addition of glucose had not increased the oxygen uptake. Hence, the starved cells were used in glucose utilization. Glucose utilization was inhibited by 30% at 10 μg of drug per ml, and the inhibition exceeded over 80% at 100 μg of miconazole per ml. Total inhibition of glucose utilization was noticed at 500 μg of miconazole per ml.

Effect of miconazole on cell permeability. The leakage of various cellular constituents like 260-nm absorbing materials, proteins, and amino acids as affected by miconazole is shown in Fig. 4. There was very little leakage of 260-nm absorbing materials when cells were exposed to 25 μg of drug per ml. Concentrations above 25 μg/ml, however, brought about leakage, and it increased with time. But the maximum leakage occurred during the first 30 min of drug exposure. Appreciable amounts of leak-

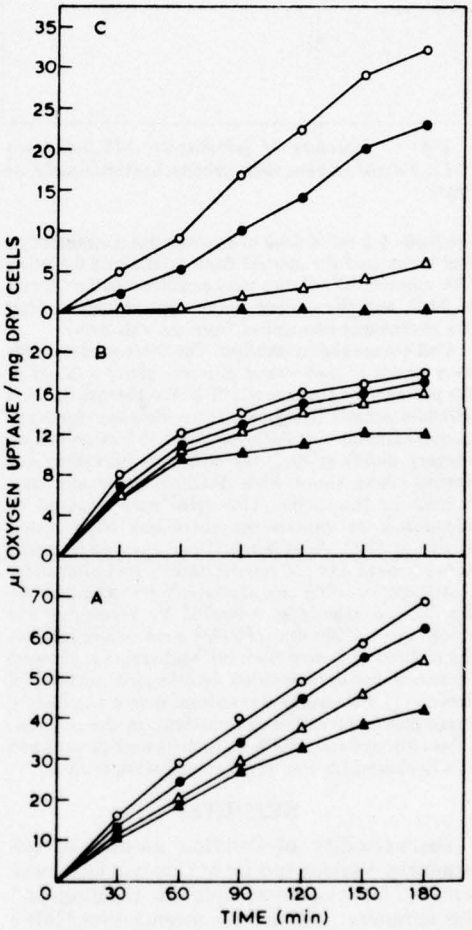

FIG. 3. *Effect of miconazole on respiration of C. albicans. Endogenous respiration of unstarved cells (A) and starved cells (B). Exogenous respiration (glucose)(C). Symbols:* ○, *control;* ●, *10 μg/ml;* △, *100 μg/ml;* ▲, *500 μg/ml.*

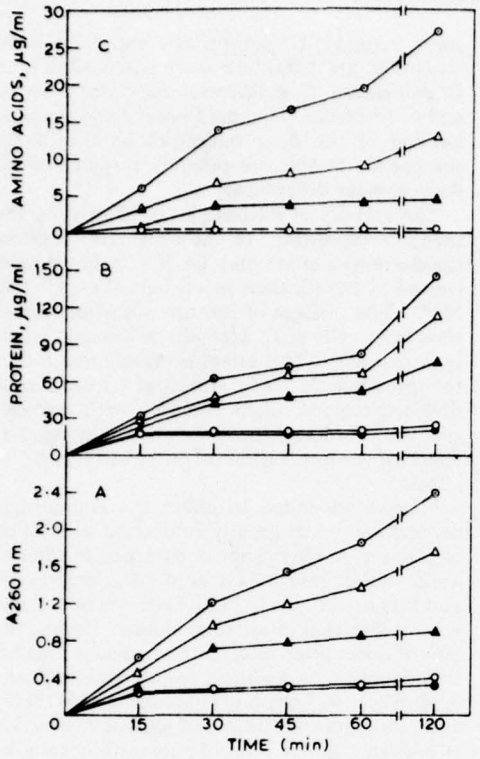

FIG. 4. *Effect of miconazole on the leakage of 260-nm absorbing materials (A), proteins (B), and amino acids (C) from the cells of C. albicans (1.5 × 10⁸ cells/ml). Symbols: ●, control; ○, 25 µg/ml; ▲, 50 µg/ml; △, 75 µg/ml; ⊙, 100 µg/ml.*

age continued to occur as the time of exposure to drug was prolonged. The leakage of proteins (B) also showed the same pattern as 260-nm absorbing materials. But at higher concentrations, the leakage increased with time, and the maximum leakage occurred within 30 min of drug exposure. Leakage of amino acids (C) also followed the same pattern as in the case of 260-nm absorbing materials and proteins.

The effect of drug on the leakage of potassium and sodium is shown in Fig. 5. The leakage of these two cations showed different patterns. Miconazole failed to cause any leakage of K^+ ions at 10 and 25 µg/ml. At 50 µg/ml the drug brought about marked efflux of K^+ ions. A small amount of Na^+ ions came out from cells of *C. albicans* in the absence of drug. In the concentration range of 10 to 25 µg/ml, the drug enhanced this leakage by two- to three-fold. Further increase in the drug concentration failed to bring about increased leakage.

The leakage of ^{32}P-labeled cellular constituents is shown in Table 3. The leakage is expressed in terms of counts per minute per milligram (dry weight) cells. The total ^{32}P taken up and incorporated by the cells during growth amounts to 3×10^5 counts per min per mg (dry weight) cells. From this data the percentage of leakage of ^{32}P-labeled cellular constituents was calculated. There was a very little leakage of labeled constituents in the absence of the drug. At 10 µg/ml the leakage was increased by 10-fold. Higher concentrations brought about

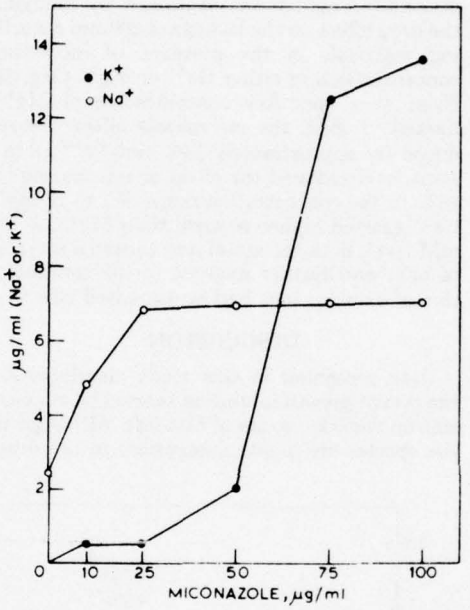

FIG. 5. *Leakage of Na^+ and K^+ from the cells of C. albicans (2.5×10^8 cells/ml) exposed to miconazole for 2 h.*

TABLE 3. *Effect of miconazole on the leakage of ^{32}P-labeled cellular constituents from the cells of C. albicans*

Miconazole (µg/ml)	Radioactivity (counts/min) in exudates from 1 mg (dry wt) of cells[a]	Leakage of ^{32}P-labeled compounds (%)
0	130	0.04
10	1,340	0.45
100	18,000	6.0
250	124,000	41.0
500	171,500	57.0
1,000	182,400	61.0

[a] Radioactivity of 3×10^5 counts per min per milligram (dry weight) of cells before exposure to drug.

an increasing amount of leakage. The leakage of 57% of the labeled constituents occurred at 500 μg/ml. A further increase of the drug by twofold, i.e., 1,000 μg/ml, has resulted in only slight increase in the effect.

Influence of divalent cations on miconazole effect. In synthetic medium where divalent cations like Mg^{2+} and Ca^{2+} are present, the growth inhibition of miconazole is less as compared in Sabouraud medium (Fig. 2). Whether these divalent ions have any effect on the miconazole action was examined by studying the drug effect on the leakage of 260-nm absorbing materials in the presence of increasing concentrations of either Ca^{2+} or Mg^{2+} (Fig. 6). Even at a very low concentration of Mg^{2+}, namely, 1 mM, the miconazole effect was reduced by approximately 34% and Ca^{2+} at the same level reduced the effect of miconazole by 46%. In the concentration range of 1 to 10 mM, Ca^{2+} exerted higher reversal than Mg^{2+}. At 10 mM level, both the metal ions caused a reversal of 82%, and further increase in the concentration of divalent ions had no increased effect.

DISCUSSION

Data presented in this study clearly reveal the potent growth inhibition exerted by miconazole on various species of *Candida*. Although all the species are highly susceptible to the drug, some, namely, *C. parapsilosis* and *C. pseudotropicalis*, are 1,000-fold more susceptible than *C. pelliculosa*, *C. guilliermondii*, *C. intermedia*, and *C. tropicalis*. The rapid uptake and stronger binding of the drug molecules by the highly susceptible strains are possibly responsible for these species differences.

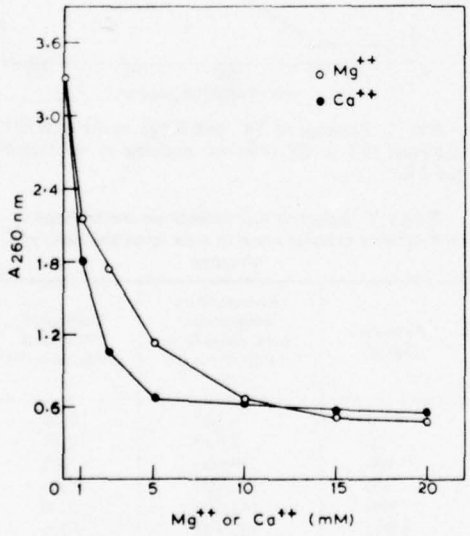

FIG. 6. *Reversal by Mg^{2+} and Ca^{2+} of the leakage of 260-nm absorbing materials from the cells of C. albicans (1.1×10^8 cells/ml) exposed to miconazole at a concentration of 100 μg/ml (0.2 mM).*

The activity of miconazole is affected by the media composition. In the synthetic medium, the decreased effect may be due to the reversal caused by the divalent metal ions like Ca^{2+} and Mg^{2+}. The leakage of 260-nm absorbing materials from cells of *C. albicans* is brought about by miconazole. This effect is greatly reduced in the presence of Ca^{2+} and Mg^{2+} ions. These divalent cations might compete with miconazole for binding sites, thereby reducing the effective concentration of drug to exert its effects.

Miconazole failed to affect the endogenous respiration, while greatly inhibiting utilization of glucose. A wide range of compounds such as amino acids, intermediates of citric acid cycle, and fats are known to be the sources of endogenous respiration in microorganisms. Hence, the lack of miconazole effect on endogenous respiration suggests its inability to interfere with the oxidative metabolism of these substrates. However, its potent inhibition of glucose utilization is probably at the level of substrate uptake by damaging the integrity of the cell membrane.

Literature is extensive in the area of membrane active antibacterial (1, 3, 7, 10, 14) and antifungal agents (8, 15). By affecting the membrane integrity and functions, they produce an initial rapid loss of high- and low-molecular-weight metabolites from the metabolic pool within the cell. Compounds like phenolics also initiate autolytic enzyme activity which cause extensive breakdown of proteins and nucleic acids. The hydrolytic products of these leak out from the cells in greater concentration. Antifungal antibiotics like polyenes specifically combine with sterol components in the membrane of susceptible organisms, resulting in the structural disruption of membrane and the loss of essential metabolites from the cell. The data presented here on miconazole show that the effect is on the cell membrane of the organism. There is very little autolytic action in the untreated cells. But miconazole brings about rapid loss of essential cellular constituents like proteins, amino acids, nucleotides and also monovalent cations. Its effect on membrane is also reflected in glucose utilization. The fact that miconazole is active on a wide range of gram-positive and gram-negative bacteria, besides yeasts and dermatophytes,

clearly reveals its binding to components present in all these structurally divergent microbes. The effect of miconazole is probably not by binding to a specific cell membrane component like ergosterol, the site where polyenes bind to exert their effect.

Antifungal agents exist which affect the cell membrane and its permeability, although their primary site of action has been proved to be elsewhere. Thus, lomofungin (6) inhibits the uptake of uracil and thymidine, and pyrrolnitrin (11) causes the leakage of 260-nm absorbing materials from the cells.

The present studies suggest the effect of miconazole on cell membrane; to exclude site(s) other than membrane, further studies are required.

ACKNOWLEDGMENTS

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STUDIES ON THE MECHANISM OF ACTION OF MICONAZOLE—II. INTERACTION OF MICONAZOLE WITH MAMMALIAN ERYTHROCYTES

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Abstract—Interaction of miconazole, an antifungal agent, with mammalian erythrocytes has been studied. Miconazole brings about hemolysis of sheep erythrocytes and 50 per cent hemolysis is observed at a drug concentration of $1{\cdot}58 \times 10^{-4}$ M. The drug-induced hemolysis is dependent on drug:cell ratio. Erythrocytes from different species do not show any significant variation in their sensitivity to miconazole. The uptake of miconazole by erythrocytes is very rapid and major portion of the drug taken up is associated with the cell membrane. Miconazole binds mostly to membrane lipoprotein fractions containing a lipid:protein ratio of 1·0. Miconazole-induced hemolysis is inhibited by serum and the serum components responsible for the inhibition have been identified as albumin and IgG. Bovine serum albumin is found quite effective in protecting erythrocytes against drug-induced hemolysis. The hemolytic activity of miconazole has been compared with polyene antibiotics, digitonin and 2-phenethylalcohol. From these results it is concluded that miconazole interacts directly with the red cell membrane and alters its permeability.

Miconazole nitrate [1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy) phenethyl)imidazole nitrate] has a broad-spectrum of activity against most pathogenic fungi and Gram-positive bacteria [1–3]. Its effective therapeutic use as a topical applicant in treating skin and nail infections and in vaginal candidiasis has been well documented [4–8].

Biochemical and electron microscopic studies on the mechanism of action of miconazole have been reported [9–11]. The drug caused a significant increase in membrane permeability in cells of *Candida albicans* as evidenced by a rapid loss of intracellular materials [9]. At low concentrations, miconazole selectively inhibited the uptake of purines and glutamine by the cells of *C. albicans* [10]. Electron microscopic examination of *C. albicans* cells exposed to miconazole revealed that the earliest drug-induced alterations are seen at the plasma membrane before any other cytoplasmic organelle seems to be involved [11]. These findings clearly reveal that miconazole impairs membrane function by inducing selective permeability changes in the cell membranes of sensitive cells.

In an attempt to determine whether the action of miconazole on membrane permeability is restricted to yeast cells or has a similar action on animal cells, its effect on the permeability of sheep erythrocytes is investigated. The present paper reports a study of various aspects of interaction of miconazole with red blood cells.

MATERIALS AND METHODS

Materials. Crystalline bovine serum albumin, cholesterol, digitonin and sodium dodecyl sulphate were purchased from Sigma Chemical Co., St. Louis, U.S.A. Amphotericin B and nystatin were kindly donated by E. R. Squibb and Sons, Inc., Princeton, U.S.A. Miconazole nitrate was a gift sample from Ethnor Ltd., Bombay, India. [^{3}H]Miconazole (sp. act. 292·3 mCi/m-mole) was a kind donation of Janssen Pharmaceutica, Belgium.

Erythrocyte preparation. Fresh blood from healthy human adults, wistar rats, guinea pigs, rabbits and sheep (for non-nucleated erythrocytes) or from chickens (for nucleated erythrocytes) were obtained using Alsever's solution as an anticoagulant. Immediately after collection, the blood was centrifuged at 1500 *g* in a Sorvall centrifuge model RC-2B for 10 min at 4° and the plasma and the buffy coat were removed. The packed erythrocytes were washed four times with 0·15 M NaCl (isotonic saline) followed by centrifugation. The cells after the last wash were suspended in 0·15 M NaCl to give an erythrocyte suspension containing approximately $1{\cdot}5 \times 10^9$ cells/ml (by hemocytometer count). Unless otherwise specified, all experiments were done with this cell suspension. Aliquots of the cell suspension were pre-incubated for 15 min at 37° before the start of the reaction.

Measurement of hemolysis. Hemolysis was determined by the addition of 0·1 ml of washed erythrocytes to 4·9 ml of 0·15 M NaCl (preincubated at 37° for 15 min) containing miconazole (in 50% ethanol) at various concentrations. Each sample had a final volume of 5 ml and erythrocyte concentration of 3×10^7 cells/ml. Control tubes containing appropriate quantity of ethanol were included in each experiment and the final concentration of ethanol in all the samples was 1 per cent. After the required time of incubation at 37°, the tubes were centrifuged and the hemoglobin released was determined by measuring the absorbance of the supernatant at 540 nm in a Beckman model-DU spectrophotometer. The data were corrected for the release of hemoglobin

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observed in controls. To determine the total amounts of intracellular hemoglobin, 0·1 ml of cell suspension was lysed in 4·9 ml of distilled water and centrifuged. The supernatant had an absorbancy of 0·800 at 540 nm. Loss of hemoglobin was expressed as a per centage of total cellular content of hemoglobin in the untreated cells.

Uptake of [^{3}H]miconazole. [^{3}H]Miconazole (sp. act. 292·3 mCi/m-mole) was diluted with unlabeled miconazole to achieve the desired molar concentration of the drug. This diluted solution (4×10^{-4} M) was used to measure the uptake of miconazole at different concentrations in 0·15 M NaCl–0·01 M sodium phosphate buffer, pH 7·4. Erythrocytes ($1·6 \times 10^8$ cells) were incubated at 37° with different concentrations of [^{3}H]miconazole dissolved in 50% ethanol. The final volume of each sample was 4 ml and contained 4×10^7 cells/ml. After 15 min of incubation, 2-ml aliquots of cell suspension were removed and centrifuged. Aliquots (100 μl) of supernatant fraction and total cell suspension were spotted on Whatman 3 MM filter squares, dried under infrared lamp, and counted in Beckman LS-100 liquid scintillation counter using 10 ml scintillation fluid containing 0·4% PPO and 0·005% POPOP in toluene. The difference between the counts in the supernatant and total cell suspension was taken as the amount of drug taken up by the cells.

The amount of miconazole associated with the cytoplasm and bound to the cell membrane was determined in the following way. Cell suspensionn incubated with [^{3}H]miconazole (1×10^{-5} M) was washed twice with 0·15 M NaCl and were then hemolysed in 5 mM sodium phosphate buffer, pH 7·4. An aliquot (100 μl) of the hemolysate was removed for counting and the remainder was centrifuged at 20,000 g for 20 min to separate the red cell membrane from the cytoplasm. Radioactivity in 100 μl of the supernatant fraction (cytoplasm) was determined as described above. The difference between the amount of drug taken by the cells and the amount in the cytoplasm is taken as the amount of drug bound to membranes.

Preparation of erythrocyte membranes. Sheep erythrocyte membranes were prepared by the procedure of Dodge *et al.* [12]. The cells were washed thrice with 0·15 M NaCl by centrifugation at 1500 g for 10 min at 4°. Membranes were prepared by the osmotic lysis of washed erythrocytes by adding 10 volumes of cold 6·5 mM sodium phosphate buffer, pH 7·4 to 1 volume of packed cells and mixed with a magnetic stirrer for 15 min. The hemolysate was centrifuged at 30,000 g at 4° for 40 min in a Sorvall centrifuge model RC-2B. Following centrifugation, the post hemolytic residue was washed four to six times with the same buffer and a final wash with isotonic saline. The milky membrane preparation thus obtained from the last centrifugation, was suspended in isotonic saline to a protein concentration of 13 mg/ml.

Solubilization of erythrocyte membranes and gel filtration. Solubilization of erythrocyte membranes and subsequent gel filtration was done according to the method of Zimmer *et al.* [13]. Membrane preparation containing 9 mg protein was incubated at 37° with 0·55 ml of 1% sodium dodecyl sulphate for 30 min. The concentration of sodium dodecyl sulphate was about 0·6 mg/mg of membrane protein. 0·015 μmoles of [^{3}H]miconazole contained in 0·15 M NaCl was then added and the final volume was made up to 2·5 ml with 0·15 M NaCl. Incubation was continued for a further 30 min and the solubilized membranes were gel filtered.

Sephadex G-100 column (48 × 1·2 cm) was equilibrated with 0·5 M NaCl–0·01 M Tris–HCl, pH 8·4. The dissolved membranes were layered onto the column and gel filtration was carried out at 28°. Fractions of about 1·5 ml were collected. The fractions comprising within the peak at 280 nm were analysed for protein, lipid and radioactivity. Protein, phospholipid and cholesterol were determined in 0·1–0·2-ml samples. Radioactivity in 0·1-ml sample was determined as described above.

Incubation of serum with [^{3}H]miconazole and gel filtration. One ml sheep serum was incubated with [^{3}H]miconazole (0·025 μmoles) at 37° for 30 min and gel-filtered at 28° on Sephadex G-200 column (53 × 2 cm) equilibrated with 0·1 M NaCl–0·05 M sodium phosphate buffer, pH 7·4. Fractions of 3 ml were collected and absorbancy at 280 nm and radioactivity determined.

Analytical procedures. Protein was determined by the procedure of Lowry *et al.* [14] with crystalline bovine serum albumin as standard. Membrane lipids were isolated by the method of Bligh and Dyer [15]. Lipid phosphorous was determined by a modification of the method of Bartlett [16], as reported by Marinetti [17] and phospholipid was estimated by multiplying the lipid phosphorus content by 25. Cholesterol was determined by the method of Glick *et al.* [18].

RESULTS

Miconazole-induced hemolysis. The time course of miconazole-induced hemolysis of sheep erythrocytes is shown in Fig. 1a. At a miconazole concentration of 1×10^{-4} M, the hemolysis by miconazole is very rapid and the rate is linear up to 20 min. Complete hemolysis resulted in 40 min. In control tubes containing ethanol at a final concentration of 1% (concentration equivalent to those in experimental tubes) hemolysis is less than 0·5 per cent.

The effect of miconazole concentration on the initial rate of hemolysis is shown in Fig. 1b. The cells are incubated with various concentrations of drug for 5 min at 37°. The extent of hemolysis is markedly influenced by the drug concentration. Up to a drug concentration of $1·2 \times 10^{-4}$ M the rate of hemolysis is very slow and thereafter increases rapidly. The drug concentration required for 50 per cent hemolysis is $1·58 \times 10^{-4}$ M and complete hemolysis occurs at a concentration of $2·2 \times 10^{-4}$ M.

The loss of hemoglobin from the erythrocytes is dependent not only on the concentration of miconazole in the incubation medium, but also on the number of erythrocytes present in the suspension. When miconazole concentration is held constant, and the number of cells per unit volume of suspending medium is increased, the hemolysis is progressively decreased with increasing cell concentration (Fig. 2). The hemolysis is only 60 and 20 per cent at erythro-

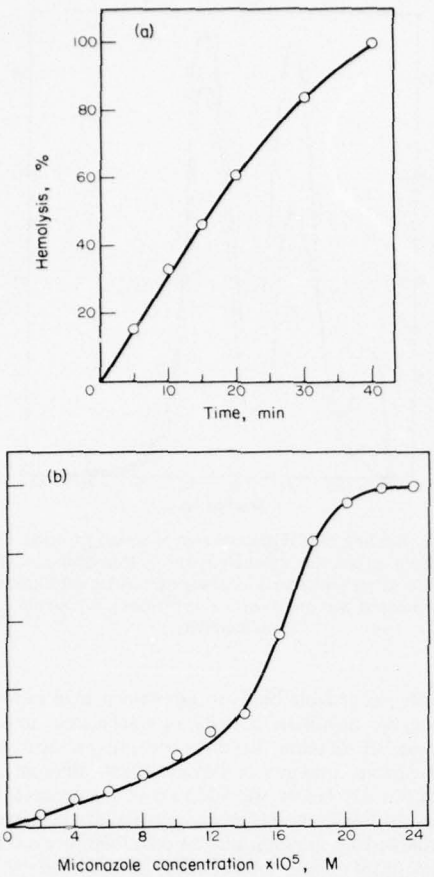

Fig. 1. Miconazole-induced hemolysis of sheep erythrocytes. (a) Time course of hemolysis of sheep erythrocytes by miconazole. Miconazole concentration, 1×10^{-4} M. (b) Effect of miconazole concentration on the initial rate of hemolysis of sheep erythrocytes. Incubation time, 5 min at 37°.

cyte concentration of 3×10^7 and 6×10^7 cells/ml, respectively.

Uptake and distribution of miconazole. When erythrocytes are incubated with [^{3}H]miconazole at non-hemolytic concentrations for 15 min at 37°, the uptake of drug by erythrocytes is very rapid and is linear over the concentrations tested (Fig. 3). Measurements of its uptake by red cells at different concentrations show that more than 60 per cent of the drug added to the red cell suspension is associated with the red cells. The distribution of radioactivity in cellular fractions of erythrocytes incubated with [^{3}H]miconazole was studied. The major portion of the radioactivity taken up by the cells (83%) is found associated with the cell membranes.

Binding of [^{3}H]miconazole to erythrocyte membrane fraction. When sheep erythrocyte membranes are solubilized with sodium dodecyl sulphate and gel filtered on Sephadex G-100, the partial separation of membrane lipoproteins is achieved. As the gel filtration proceeds, the lipid:protein ratio changes from above 1 to about 0·2. This separation of membrane

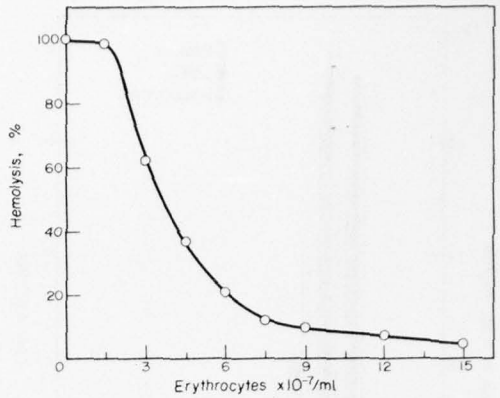

Fig. 2. Effect of sheep erythrocyte concentration on hemolysis by miconazole. Miconazole concentration, 1×10^{-4} M.

lipoproteins indicate that the lipoproteins with the higher lipid content are eluted first. To characterize the miconazole binding site on membranes the solubilized membranes are incubated with [^{3}H]miconazole and gel-filtered. The pattern of elution of lipoproteins and radioactivity are shown in Fig. 4. The radioactivity peak coincided with the membrane lipoprotein peak having a lipid:protein ratio of 1·0. About 90 per cent of the total radioactivity present during the incubation of membranes with the drug got eluted with the liproprotein fractions indicating a strong binding of miconazole to membrane components.

Inhibition of hemolysis by serum. The effect of homologous serum on the miconazole-induced hemolysis of sheep erythrocytes is shown in Fig. 5. The erythrocytes are preincubated with serum and the reaction is started by the addition of miconazole to a final concentration of 1×10^{-4} M and incubated for 20 min at 37°. The serum has a protective action and inhibits the miconazole-induced hemolysis even at low concentrations. At 0·2% serum level, the hemolysis is inhibited by 60 per cent and at 1% the inhibition is more than 90 per cent.

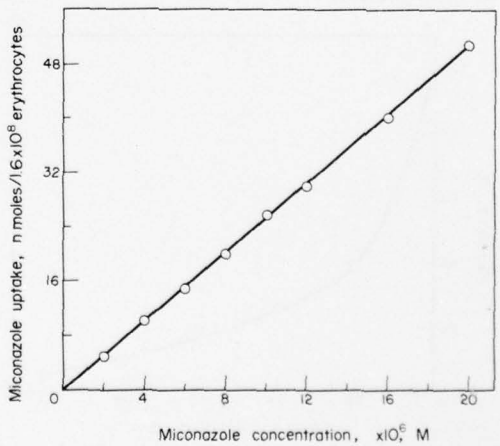

Fig. 3. Uptake of [^{3}H]miconazole by sheep erythrocytes. Incubation time, 15 min at 37°.

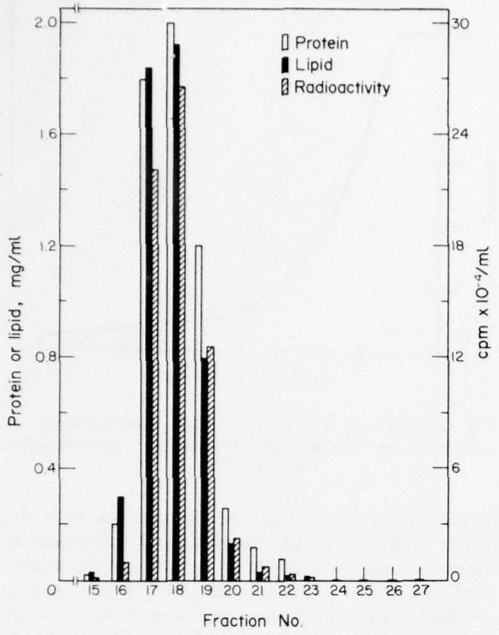

Fig. 4. Binding of [^{3}H]miconazole to erythrocyte membrane lipoprotein fractions. Erythrocyte membranes, solubilized with sodium dodecyl sulphate, were incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-100 column. After gel filtration, individual fractions were analysed for radioactivity, protein and lipid content.

To characterize the serum component(s) responsible for the inhibition of miconazole-induced hemolysis, serum is incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-200 (Fig. 6). The radioactivity of the drug eluted in fractions containing IgG and albumin. About 40 per cent of the total radioactivity is found in IgG fraction and 60 per cent in the albumin. These results indicate that miconazole binds to serum proteins and the inhibitory effect of serum on hemolysis is a consequence of miconazole binding to serum proteins.

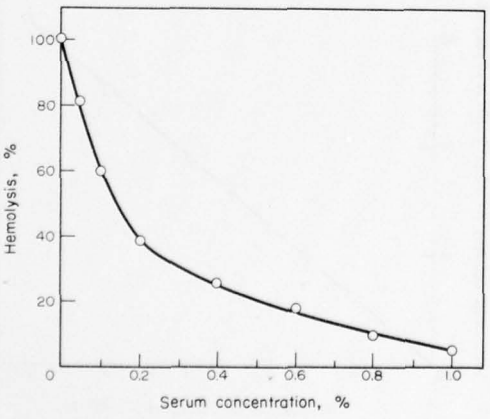

Fig. 5. Inhibition of miconazole-induced hemolysis of sheep erythrocytes by homologous serum. Miconazole concentration, 1×10^{-4} M.

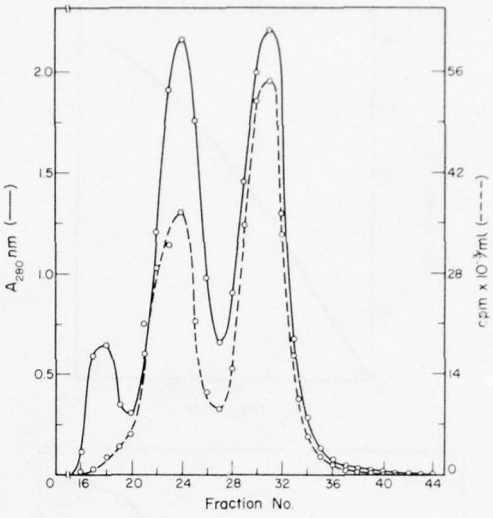

Fig. 6. Binding of [^{3}H]miconazole to serum proteins. One ml sheep serum was incubated with [^{3}H]miconazole and gel-filtered on Sephadex G-200 column. After gel filtration, each fraction was analysed for absorbancy at 280 nm and radioactivity.

Since miconazole binds to albumin it is of interest to see the hemolytic activity of miconazole in the presence of albumin. Erythrocytes are preincubated with various amounts of bovine serum albumin for 30 min at 37° before the addition of miconazole. As shown in Fig. 7 the hemolytic activity of miconazole is inhibited by albumin and at an albumin concentration of 0·4 mg/ml, the hemolysis is inhibited by 68 per cent. The hemolytic activity of miconazole is partially retained at 2 mg/ml albumin concentration.

Relative hemolytic effect of miconazole compared with other membrane-active drugs. For comparison, the effect of other membrane-active agents like polyene antibiotics, digitonin and 2-phenethylalcohol on sheep erythrocytes is studied. The cells are incubated with the different concentrations of each drug for 5 min at 37° and from the graphic plot of the

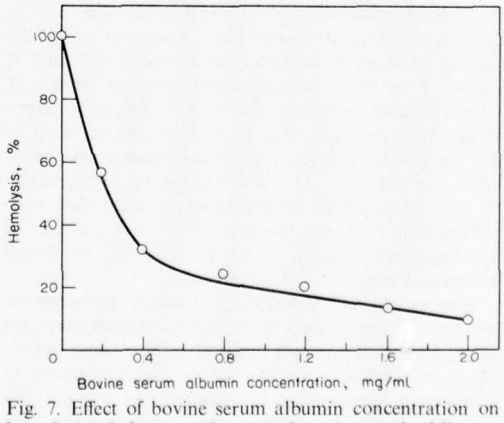

Fig. 7. Effect of bovine serum albumin concentration on hemolysis of sheep erythrocytes by miconazole. Miconazole concentration, 1×10^{-4} M.

Table 1. Comparative hemolytic effect of miconazole with other membrane active agents

Drugs tested	Concentration of drug required for 50% hemolysis
Amphotericin B	9.2×10^{-6} M
Nystatin	8.4×10^{-5} M
Digitonin	2.6×10^{-6} M
2-Phenethylalcohol	5.7×10^{-2} M
Miconazole	1.58×10^{-4} M

To tubes containing different concentrations of drugs indicated in the table, washed sheep erythrocytes were added to a final concentration of 3×10^7 cells/ml, incubated for 5 min at 37° and immediately centrifuged. The hemoglobin released was measured as described under Materials and Methods. Amphotericin B and nystatin were dissolved in dimethylformamide, digitonin in absolute alcohol and dilutions of 2-phenthylalcohol in 50% ethanol. The control tubes contained the equivalent amount of solvents in which the drug was dissolved.

data, the concentration of each drug needed to bring about 50% hemolysis is calculated. As shown in Table 1, digitonin is a more potent hemolytic agent while 2-phenethylalcohol is least effective. Digitonin, amphotericin B and nystatin cause 50% hemolysis at 60, 17 and 1·8-fold lesser concentration than miconazole, respectively. On the other hand, 2-phenethylalcohol requires about 360-fold higher concentration than miconazole to bring about 50% hemolysis.

Hemolytic effect of miconazole on erythrocytes from different species. Erythrocytes from different species are examined for their susceptibility to miconazole (Table 2). The washed erythrocytes from different species are incubated with different concentrations of miconazole for 5 min at 37° and from a graphic plot of the data, the hemolytic value corresponding to 50% hemolysis is calculated. Among the non-nucleated erythrocytes, the variation in their susceptibility to miconazole-induced hemolysis is not very significant. Erythrocytes from hamster require comparatively lower concentration (0.9×10^{-4} M) than erythrocytes from humans which require a 3-fold higher concencentration (2.76×10^{-4} M). Erythrocytes from guinea pig, rabbits and sheep are susceptible to the same extent and require about 1·7-fold lower concentration than human erythrocytes. The nucleated erythrocytes from chicken are also equally sensitive to the action of miconazole and 50% hemolysis occurs at a drug concentration of 1.48×10^{-4} M.

Table 2. Hemolytic effect of miconazole on erythrocytes from different species

Erythrocyte source	Concentration of miconazole required for 50% hemolysis ($\times 10^{-4}$ M)
Rat	1·18
Rabbit	1·58
Guinea pig	1·54
Hamster	0·90
Sheep	1·58
Human	2·76
Chicken	1·48

The washed erythrocytes (3×10^7 cells/ml) from different species were incubated with different concentrations of miconazole for 5 min at 37° and centrifuged immediately. Hemoglobin released was measured as described under Materials and Methods. From the graphic plot of the data, the concentration of drug required for 50% hemolysis was calculated.

DISCUSSION

The drug-induced hemolysis of red cells is currently thought to occur by either of two basic mechanisms [19–22]. The first involves direct interaction of the drug with the red cell membrane which results in changes in membrane structure, increased permeability, osmotic swelling and hemolysis. In the second mechanism, the drug first penetrates into the cell interior where it interferes with cellular metabolism ultimately resulting in membrane damage and hemolysis. The drug-induced hemolysis in the second mechanism may be due to enzyme deficiencies, unstable hemoglobins or immune mechanisms [20, 21].

The present findings reveal that miconazole has a profound effect on erythrocyte membrane structure and brings about rapid hemolysis. This raises the possibility that hemolysis is the result of a direct interaction between miconazole and the plasma membrane of the erythrocytes. A prerequisite to direct alteration of membrane permeability is that miconazole must interact with the cell membrane. Data presented in this paper show that [^{3}H]miconazole at low, non-hemolytic concentrations binds largely to the red cell membrane and thus satisfying a necessary condition.

Identification of the binding site on the erythrocyte membrane surface would help for a better understanding of the mechanism of action of membrane-active drugs. In our effort to characterize the miconazole-binding site on the erythrocyte membrane, it has been shown that miconazole binds to membrane lipoproteins strongly and that miconazole binding is maximum in lipoprotein fraction containing lipid: protein ratio of 1·0. The concentration of sodium dodecylsulphate (0·6 mg SDS/mg membrane protein) employed to solubilize the membrane in the present experiment does not cause any loss of biological activity of the membrane [13, 23], and no disruption of lipoprotein structure of the membrane is evident (Fig. 4). The present experiments, however, do not indicate, the differential binding of miconazole to either lipid or protein part on the membrane.

The protective effect of serum against miconazole-induced hemolysis apparently results from the binding of miconazole to serum proteins. Serum albumin is found quite effective in protecting erythrocytes against miconazole-induced hemolysis. The binding of miconazole to serum proteins would result in a reduction of effective drug concentration to bring about hemolysis of erythrocytes. Several drugs are known to bind to albumin in the serum, but the binding of miconazole to IgG, presented in this paper, raises two possibilities. It may be either due to the binding of miconazole to IgG itself or to the elution of albumin dimers formed in the serum along with IgG. In the latter case the drug primarily binds to albumin, but due to the formation of albumin dimers the radioactivity appears in the IgG peak.

Polyene antibiotics, which have been extensively studied for their membrane damaging properties,

have been compared with miconazole for their hemolytic activity. Among the polyenes, amphotericin B showed higher hemolytic effect than miconazole and nystatin showed almost equal effect. Digitonin, a plant saponin, exerts higher hemolytic activity than miconazole. Polyenes and saponins induce membrane damage by their strong binding to cholesterol in the membrane [24–32]. 2-Phenethylalcohol, which is known to impair the cell membrane function in bacteria [33, 34], yeasts [35], fungi [36], tumor cells [37] and mammalian erythrocytes [38], showed lower hemolytic activity than miconazole.

There is little variation in the hemolytic effect of miconazole on erythrocytes from different species and it is independent of the presence or absence of a nucleus. The basis for the reported differences in the hemolytic tendency among species is not known but variation in the protein and lipid composition of the red cell membrane may account for such differences [39–46].

On the basis of the results presented in this paper we conclude that miconazole interacts directly with the red cell membrane and brings about permeability alterations. Though miconazole binds to membrane lipoproteins strongly, the nature of this interaction is not known. The chemical nature of the drug indicates a possible mode of interaction. Since the drug is hydrophobic in nature, the interaction may be of a hydrophobic type and this would lead to an impairment of membrane function. Further studies are needed to elucidate this aspect.

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46

Mechanism of Action of Miconazole: Labilization of Rat Liver Lysosomes In Vitro by Miconazole

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Miconazole, a potent antifungal agent, labilizes rat liver lysosomes. Its labilizing effect is followed by measuring the release of lysosomal hydrolases, namely, acid phosphatase, β-glucuronidase, and arylsulfatase A. The effect of miconazole is concentration dependent in the range of 10^{-5} to 1.2×10^{-4} M. However, at higher concentrations, miconazole inhibits enzyme release but does not inhibit enzyme activities per se. The effect of miconazole depends on the drug/lysosome ratio and is influenced by the pH of the incubation media, being minimal at alkaline pH. Membrane-active drugs such as nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin have been compared with miconazole for their lysosome-labilizing action. The effect of miconazole on the lysosomal membrane is confirmed by a decrease in turbidity of the lysosomal suspension.

Miconazole {1-[2,4-dichloro-β-(2,4-dichlorobenzyloxy)-phenethyl]imidazole nitrate} has a broad spectrum of antimicrobial activity against pathogenic and nonpathogenic yeasts, dermatophytes, numerous saprophytic fungi, and gram-positive bacteria (13, 25, 31). Chemotherapeutic activity of miconazole as a topical applicant is well documented in the treatment of skin and nail infections and vaginal candidiasis (2, 5, 14, 29, 32). Studies on the mechanism of biological action of miconazole have been recently reported (9, 10, 28, 33; K. H. Sreedhara Swamy, M. Sirsi, and G. Ramananda Rao, Biochem. Pharmacol., in press). The drug induces leakage of intracellular materials from cells of *Candida albicans* (28), and at low concentrations it selectively inhibits the uptake of purines and glutamine into these cells (33). Electron microscopic examination of cells of *C. albicans* exposed to miconazole revealed that the earliest drug-induced alterations are seen at the plasma membrane (9, 10). Further, miconazole has been shown to induce hemolysis of mammalian erythrocytes and binds strongly to erythrocyte membrane lipoproteins (Sreedhara Swamy et al., in press). These investigations clearly indicate that the paramount feature of the biological action of miconazole is its interaction with cell membrane of sensitive organisms, resulting in the impairment of membrane function and eventually cell death.

In an attempt to delineate the mode of action of miconazole on cellular and organellar membranes and to obtain a more comprehensive view of biological action, we have carried out studies on the interaction of miconazole with rat liver lysosomes. The present paper describes the effect of miconazole on the integrity of lysosomal membrane, providing further evidence that miconazole interacts with biological systems by impairing membrane function.

MATERIALS AND METHODS

Chemicals. Miconazole nitrate was a gift sample from Ethnor Ltd., Bombay, India. Hexachlorophene, digitonin, 2-phenethylalcohol, *p*-nitrophenylphosphate, *p*-nitrocatechol sulfate, phenolphthalein-β-D-glucuronide, Triton X-100, and tris(hydroxymethyl)-aminomethane (Trizma base) were purchased from Sigma Chemical Co., St. Louis, Mo. Nystatin was kindly donated by E. R. Squibb and Sons, Inc., Princeton, N.J. All other chemicals were of analytical reagent grade.

Preparation of rat liver lysosomes. Inbred Wistar A/Iisc rats weighing 100 to 120 g were killed by cervical dislocation, and the liver was quickly dissected out into ice-cold 0.15 M NaCl (isotonic saline). The liver was washed twice with 0.15 M NaCl, weighed, minced finely with scissors, and suspended in 0.25 M sucrose. The liver was homogenized in 0.25 M sucrose (5 ml of solution per g of liver) using a Potter-Elvehjem glass homogenizer with a motor-driven Teflon pestle. The homogenate was first centrifuged at $1,500 \times g$ in a Sorvall centrifuge, model RC 2-B, for 10 min at 4 C to sediment unbroken cells and nuclei. The supernatant was then centrifuged at $20,000 \times g$ for 30 min, and the pellet containing the lysosomes was suspended gently in 0.25 M sucrose to give a final concentration of 10 mg of protein per ml.

Effect of miconazole on rat liver lysosomes. The effect of miconazole on lysosomes was followed by measuring the release into the medium of lysosomal hydrolases. Rat liver lysosomes (0.5 mg of protein per ml) were incubated in 0.25 M sucrose containing

miconazole (dissolved in 50% ethanol) at various concentrations for 15 min at 37 C. All incubation mixtures, including controls, contained ethanol at a final concentration of 1%. After the incubation, the tubes were chilled in ice and centrifuged at 20,000 × *g* for 20 min, and the resulting supernatants were assayed for acid phosphatase, β-glucuronidase, and arylsulfatase A. The enzyme activity in the supernatant is expressed as percentage of total activity obtained in the presence of 0.1% Triton X-100. The data were corrected for the release of enzymes in control samples.

Enzyme assays. Acid phosphatase activity was determined by the method of Igarashi and Hollander (17), using *p*-nitrophenyl phosphate as substrate.

The reaction mixture for β-glucuronidase assay in 1 ml contained 30 mM acetate buffer, pH 4.5, 0.5 ml of the supernatant, and 0.4 mM phenolphthalein-β-D-glucuronide (sodium salt). The reaction mixture was incubated at 37 C for 30 min, and the reaction was stopped by adding 5 ml of 0.2 M glycine-NaOH buffer, pH 10.4. The absorbancy of the color was measured at 540 nm.

Arylsulfatase A was estimated by the method of Jerfy and Roy (18), using *p*-nitrocatechol sulfate as substrate.

Protein was estimated by the method of Lowry et al. (22).

RESULTS

Effect of miconazole on rat liver lysosomes. The time course of miconazole-induced release of lysosomal enzymes is shown in Fig. 1. At a miconazole concentration of 5×10^{-5} M, the rate of release of acid phosphatase and arylsulfatase A showed an increase up to 40 min and thereafter remained constant. On the other hand, the release of β-glucuronidase reached maximum by 30 min and showed no further significant increase up to 60 min of incubation. The release of acid phosphatase and arylsulfatase A by miconazole at 60 min is about 44%, and that of β-glucuronidase is 32% of the total enzyme activity present in the lysosomes.

The effect of increasing concentrations of miconazole on the release of acid phosphatase, arylsulfatase A, and β-glucuronidase from lysosomes is shown in Fig. 2. The lysosomes were incubated with various concentrations of miconazole in 0.25 M sucrose for 15 min. Miconazole caused an increased release of all three lysosomal enzymes up to a concentration of 1.2×10^{-4} M, and further increase in the drug concentration showed decreasing enzyme activities in the supernatant.

The labilization of lysosomes is dependent not only on the concentration of miconazole, but also on the amount of lysosomes in the incubation medium. Increase in the lysosomal protein concentration (number of lysosomes) per unit volume of suspending medium at a constant miconazole concentration caused a progressive decrease in the release of enzymes from lysosomes (data not shown).

Influence of pH on the miconazole-induced labilization of lysosomes. The effect of miconazole on lysosomes is dependent on the pH of the incubation media (Table 1). The release of lysosomal enzymes by miconazole was equally effective at pH 5.0 (0.25 M sucrose-0.01 M acetate) and pH 6.8 (unbuffered 0.25 M su-

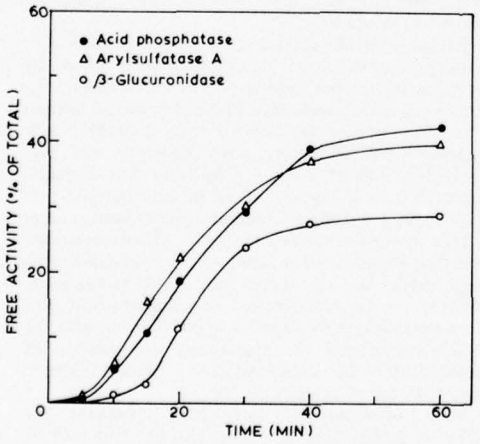

FIG. 1. *Time course of miconazole-induced lysis of rat liver lysosomes. Miconazole concentration, 5 × 10^{-5} M.*

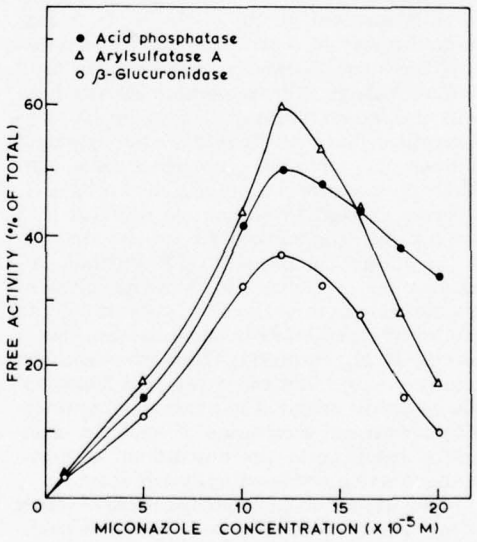

FIG. 2. *Effect of miconazole concentration on the release of lysosomal enzymes. Incubation time, 15 min at 37 C.*

TABLE 1. *Influence of pH of the incubation medium on the release of lysosomal enzymes by miconazole*[a]

Incubation medium	Free activity (% of total)					
	Acid phosphatase		Arylsulfatase A		β-Glucuronidase	
	5×10^{-5} M[b]	10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M	10^{-4} M
0.25 M sucrose-0.01 M acetate (pH 5.0)	11.6	30.8	15.8	46.2	16.0	35.7
0.25 M sucrose (pH 6.8)	17.0	44.0	17.0	43.8	12.0	32.0
0.25 M sucrose-0.01 M Tris-hydrochloride (pH 8.0)	5.0	16.5	10.3	20.9	5.5	19.4

[a] Lysosomes (0.5 mg of protein per ml) were incubated at different pH values with the indicated concentration of miconazole for 15 min at 37 C. After centrifugation at 20,000 × *g* for 20 min at 4 C, the enzyme activities released into the supernatants were determined. Total activity in each sample was measured by incubation of lysosomes with 0.1% Triton X-100. After correction for release of enzymes in controls containing 1% ethanol, the data were expressed as percentage of total enzyme activity. Tris, Tris(hydroxymethyl)aminomethane.

[b] Miconazole concentration.

crose), but the extent of release was reduced at pH 8.0 [0.25 M sucrose-0.01 M tris(hydroxymethyl)aminomethane-hydrochloride].

Decrease in turbidity of lysosomal suspension caused by miconazole. Incubation of lysosomes with miconazole in 0.25 M sucrose resulted in a decrease in the lysosomal turbidity. It was measured at 25 C by adding lysosomes to 0.25 M sucrose containing miconazole, and the absorbancy of the suspension was measured at 520 nm in a Carl-Zeiss spectrophotometer at different time intervals. The decrease in turbidity of the lysosomal suspension after 2 min was about 12 and 26% at miconazole concentrations of 5×10^{-5} M and 10^{-4} M, respectively. Under similar conditions, 0.1% Triton X-100 decreased the turbidity of lysosomal suspension by about 71%.

Lysosome labilizing action of miconazole as compared with other membrane-active drugs. For comparison, the effect of some membrane-active drugs such as nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin on rat liver lysosomes was studied (Table 2). Nystatin was relatively ineffective in releasing enzymes from lysosomes. 2-Phenethylalcohol required a very high concentration (5×10^{-2} M) to induce drastic changes in lysosomal integrity, resulting in the release of lysosomal enzymes. Both hexachlorophene and digitonin disrupted lysosomes, and at 10^{-4} M the lysosome labilization brought about by these drugs and miconazole was quite similar.

TABLE 2. *Comparative effect of miconazole with some membrane-active drugs on labilization of rat liver lysosomes*[a]

Drugs	Concn (M)	Free enzyme activity (% of total)		
		Acid phosphatase	Arylsulfatase A	β-Glucuronidase
Nystatin	1×10^{-4}	1.70	0.97	2.40
2-Phenethylalcohol	5×10^{-4}	5.30	2.90	5.80
	1×10^{-4}	2.40	1.10	2.90
	5×10^{-3}	5.40	1.60	11.70
	5×10^{-2}	59.20	38.20	25.40
Hexachlorophene	1×10^{-5}	8.10	7.40	7.80
	1×10^{-4}	35.60	34.80	58.80
Digitonin	1×10^{-5}	2.00	1.00	11.70
	1×10^{-4}	46.70	40.00	63.70
Miconazole	1×10^{-5}	4.20	4.40	3.40
	1×10^{-4}	44.00	43.80	32.00

[a] Lysosomes (0.5 mg/ml) were incubated for 15 min in 0.25 M sucrose containing different concentrations of drugs (as indicated in the table) and centrifuged at 20,000 × *g* for 20 min at 4 C. The enzyme activities in the supernatant were determined as described in Materials and Methods. Total enzyme activity was measured by incubation of lysosomes with 0.1% Triton X-100. Nystatin was dissolved in dimethylformamide. Digitonin, hexachlorophene, and miconazole were dissolved in 50% ethanol, and dilutions of 2-phenethylalcohol were made in 30% ethanol. The control samples contained solvents at concentrations present in experimental tubes.

DISCUSSION

The data presented in this paper clearly reveal that miconazole has a profound effect on lysosomal membrane and causes release of acid phosphatase, β-glucuronidase, and arylsulfatase A from lysosomes. Its effect is concentration dependent, and, when lysosomes are exposed to different concentrations of miconazole, an opti-

mum concentration for lysis is reached (1.2×10^{-4} M) instead of a saturation response (Fig. 2). The release of enzymes is reduced beyond this optimum concentration. The decreased enzyme activities in the supernatant at higher concentrations of miconazole is apparently due to its interference with release of enzymes, since the drug failed to inhibit enzyme activities per se (data not shown).

Turbidity of lysosomes often serves as an indication of their structural integrity. Miconazole decreased the turbidity of lysosomal suspension, thus providing evidence for its effect on lysosomal membrane structure.

The lysosome-labilizing effect of miconazole was compared with that of nystatin, 2-phenethylalcohol, hexachlorophene, and digitonin. Nystatin, a polyene antibiotic, impairs cell membrane function by binding to sterols in the membrane of susceptible organisms (15, 20). Nystatin is relatively ineffective in releasing enzymes from lysosomes. The data is consistent with the previous finding (34) that the high-molecular-weight group of polyenes (nystatin and amphotericin B) are least effective in disrupting lysosomes. 2-Phenethylalcohol, which is known to interact with the cell membrane of bacteria (26, 30), yeasts (6; T. K. Narayanan, Ph.D. thesis, Indian Institute of Science, Bangalore, India, 1975), fungi (21), tumor cells (4), and mammalian erythrocytes (3; Sreedhara Swamy et al., in press), requires very high concentrations to disrupt lysosomes. Earlier, 2-phenethylalcohol was shown to release acid phosphatase from chicken liver lysosomes at high concentrations (16).

Hexachlorophene and digitonin are included in the present studies because they have been shown to interact with various biological systems by impairing cell membrane function. Hexachlorophene has been shown to alter the permeability of plant (24), bacterial (8, 19, 27), and mammalian erythrocyte membranes (7, 12, 23). Digitonin, a plant saponin, induces membrane damage by binding to cholesterol in the membrane (1, 11). The results presented in this paper clearly show that both hexachlorophene and digitonin disrupt rat liver lysosomes and release enzymes. These compounds both exert a maximum release of β-glucuronidase when compared with the release of acid phosphatase and arylsulfatase A. In contrast, miconazole and 2-phenethylalcohol released β-glucuronidase to a lesser extent than did the other two enzymes. Thus, the differential effects showed by these drugs on lysosomes appear to be drug specific.

In conclusion, the present findings and the earlier studies on the action of miconazole on membranes of yeasts (9, 10, 28, 33) and mammalian erythrocytes (Sreedhara Swamy et al., in press) show that the drug interacts with both cellular and subcellular membranes.

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C.4. IMMUNITY IN DERMATOMYCOSES

4a. Development of experimental dermatomycoses in laboratory animals.

Experimental infections in laboratory animals help not only in establishing the pathogenicity of an isolated fungus but also in several other purposes, viz., to obtain the tissue phase of certain fungi like Histoplasma, Cryptococcus, etc., to determine the hair-spore relationship of an isolated fungus, to understand the role of fungi in allergy and the host-immune response and to evaluate antimycotic agents in vivo.

In order to standardise and study the course of an experimental dermatophytic infection in a susceptible host, laboratory animal species like rats, mice and guinea pigs were examined for their susceptibility to various dermatophyte species (Table 1). Guinea pigs were found to be susceptible to Trichophyton mentagrophytes var granular when infected by cutaneous scarification method. In brief the method is as follows. The animals were clipped and shaved over the flanks. The shaved skin was scarified with No.1 grit sand paper and 0.5 ml of spore suspension in saline (60mg dry weight) was rubbed-in. The peak of infection was seen by the end of 21 days and lasted for 35 days with spontaneous clinical recovery. The morphology of the lesions during various phases of infection and mycological examination of skin scales and hairs are described in Table 2. The lesions were initially boggy and erythematous, later became scaly and dry typical of a ringworm lesion. The fungus was present in skin and hair as mycelia and arthrospores. The invasion of the hair was of ectothrix type.

TABLE 1. EXPERIMENTAL DERMATOMYCOSES IN LABORATORY ANIMALS

Organisms used	Species of animals tested	Mode of infection	Results
Microsporum gypseum	Rats	Spore suspension was rubbed-in on scarified skin	Mild inflammation lasted for 2 days with no infection.
Trichophyton mentagrophytes (granular variety)	Mice	,,	Inflammation and infiltration persisted for 10 days with scab formation, which sloughed off later. No infection was established.
,,	,,	By intravenous route	Neither inflammation nor infection was established.
Trichophyton mentagrophytes (downy variety)	Guinea pigs	Spore suspension was rubbed-in on scarified skin	No infection.
,,	,,	By intradermal route	No infection.
Trichophyton mentagrophytes (granular variety)	,,	Spore suspension was rubbed-in on scarified skin	Infection

TABLE 3. DURATION AND COURSE OF REINFECTIONS IN GUINEA PIGS WITH *TRICHOPHYTON MENTAGROPHYTES*

Infection (duration in days)	Morphology of the	Mycological examination of the lesions by	
		Microscopy	Culture
First infection (35-40)	Infiltration erythema followed by abundant scales	Mycelia and spores (upto 28-30 d)	-ve (by 35-40 d)
Second infection (25-30)	,,	,, (upto 20-25 d)	-ve (by 25-30 d)
Third infection (20-25)	Infiltration, increased erythema with scales	,, (upto 15-20 d)	-ve (by 20-25 d)
Fourth infection (10-12)	,,	Mycelia in very few scales (upto 7-8 d)	-ve (by 10-12 d)

Repeated infections.

The animals which were infected and self-cured, were repeatedly infected for 4 times and the course of infection and the morphology of the lesions during each infection were examined. The results are presented in the Table 3. The results revealed that reinfections in once infected and self-cured animals led to rapid recovery and increased dermal reactions indicating the development of acquired immunity.

Immune response during the course of experimental dermatomycoses in guinea pigs.

The spontaneous cure of the primary lesions and the development of increased resistance to reinfections indicate the operation of immune mechanisms during the infection and recovery. The immune response during the infection and recovery was analysed as to the amount and types of circulating antibodies in the sera. A low titer of precipitating, agglutinating and complement fixing antibodies were detected (Table 4). The animals showed a delayed-type of hypersensitive reactions to the soluble extract of T. mentagrophytes when challenged intradermally (Table 5). The reactions appeared 2 weeks after the infection, increased thereafter and remained at high level even after clinical recovery.

4b. Analysis of immune response in guinea pigs immunized with soluble extract of T. mentagrophytes.

Analysis of immune response during the course of infection and recovery indicates that both antibody-

TABLE 2. EXPERIMENTAL DERMATOMYCOSIS IN GUINEA PIGS INFECTED WITH *TRICHOPHYTON MENTAGROPHYTES*

Days after infection	Morphology of lesions	Mycological examination of the lesions by Microscopy		Culture
		Skin scales	Hair stubs	Skin scales and hair stubs
2-4	Acute dermatitis with oozing of sero-sanguinous fluid followed by scab formation.	Nil	Nil	+
5-7	Infiltration and crust formation associated with erythema and edema.	Branched mycelia (abundant)	Nil	+
8-15	Scales all over the lesion followed by gradual alopecia in the centre.	,,	Mycelia around the hair shaft	+
16-25	Scales confined to the border with central alopecia	,,	Mycelia inside and outside the hair matrix; arthrospores in parallel chains.	+
26-35	Fewer scales in the border and hair growth in the centre	Mycelia (few)	,,	+
36-40	Gradual clinical recovery with hair growth all over the lesion	±	Nil	Nil

* Dose of infection: 60 mg (dry weight) of spore suspension (0.5 ml) per lesion.

TABLE 4. LEVELS OF ANTIBODIES IN SERA OF GUINEA PIGS INFECTED WITH TRICHOPHYTON MENTAGROPHYTES DURING VARIOUS PHASES OF INFECTION AND RECOVERY.

Types of antibodies	Early infective phase	Peak of infection	Declining phase	Clinical recovery
	on day 7	on day 21	on day 25	on day 40
	titer	titer	titer	titer
Precipitin	nil	1:4	1:8	1:4
Agglutinin	nil	1:40	1:40	1:320
Complement-fixing	nil	1:2	1:8	1:8

TABLE 5. CELL-MEDIATED IMMUNE RESPONSE IN GUINEA PIGS INFECTED WITH TRICHOPHYTON MENTAGROPHYTES.

Hypersensitivity reactions	Early infective phase	Peak of infection	Declining phase	Clinical recovery
	on day 7	on day 21	on day 25	on day 40
	diameter of induration (mm)			
Delayed-type	6 to 8	9 to 15	13 to 16	11 to 15

mediated and cell-mediated immune responses are in operation. The presence of low titers of the antibodies might probably be due to the lack of adequate antigenic stimulus. Since the organism is confined to superficial layers, the amounts of diffusible antigenic components reaching the lymphoid system might be very low. Hence the immune responses in detail were studied in actively immunized animals.

Soluble extract from acetone-washed 7-day old mycelium of T. mentagrophytes was prepared in 0.1 M phosphate buffered saline (pH 7.2). The dialyzed extract (12 mg protein) in Freund's Complete Adjuvant was injected subcutaneously in three doses at weekly intervals. One more dose was given 14 days after the third injection to boost the level of antibodies and the cellular reactions. Animals were examined for the circulating antibodies and the delayed-type hypersensitive reactions. The levels of circulating antibodies in sera of immunized animals are shown in Table 6. The titer of precipitating antibodies was doubled with a booster dose. But there was no change in other types.

The cellular immunity was studied in detail. In summary the following techniques were employed for the study and the results are shown in Table 7.

1. Delayed-type of hypersensitivity reactions in animals challenged with soluble extract of the fungus.

2. Inhibition of migration of peritoneal exudate cells from the capillaries in the presence of soluble extract.

3. Antigen recognition capacity of the sensitized

TABLE 6. LEVELS OF CIRCULATING ANTIBODIES IN GUINEA PIGS ACTIVELY IMMUNIZED WITH SOLUBLE EXTRACT OF TRICHOPHYTON MENTAGROPHYTES

Dose of antigen	Titer of antibody types		
	Precipitin	Agglutinin	Complement fixing
Soluble extract (12 mg protein)	1:16	1:1024	1:8
Booster dose (3 mg protein)	1:32	1:1024	1:8

TABLE 7. CELLULAR REACTIONS IN GUINEA PIGS ACTIVELY IMMUNIZED WITH SOLUBLE EXTRACT OF TRICHOPHYTON MENTAGROPHYTES

Skin reactions (DTH) against soluble extract	18-22 mm of induration.
Migration of peritoneal exudate cells in the presence of soluble extract	inhibited
Transformation of lymphocytes in the presence of soluble extract	15 - 20%
Antigen recognizing cells (immunocytoadherence)	19%

lymphocytes from spleen as studied by the immunocyto-adherence (ICA) test.

4. Transformation or blastogenesis of sensitized lymphocytes from spleen when exposed to soluble extract as antigen. This was studied both by microscopy and ^{3}H-thymidine incorporation during blastogenesis.

The immune sera was further tested for its biological activity on the spores of T. mentagrophytes. The germination of macroconidia of T. mentagrophytes was completely inhibited when incubated with immune sera at 10% level. Further this inhibitory action in the presence of 'complement' is being examined.

These studies indicate clearly that on immunization there is a significant increase in the titer of circulating antibodies (which are biologically active) and the cell mediated reactions.

The soluble extract used for immunization is known to contain complex molecules like lipopolysaccharides and proteins which are highly immunogenic. Lipopolysaccharides and proteins were separated by phenol-water extraction and trichloroacetic acid precipitation methods. The sera from the immunized animals were tested against these components and soluble extract for precipitin bands in gel diffusion method. The soluble extract gave 3 precipitin lines, the proteins 2 and the lipopolysaccharide fraction only one.

The capacity to evoke hypersensitivity reactions by these components was also examined by intradermal administration. The protein fraction elicited only the delayed-type of hypersensitive reaction. The

lipopolysaccharide fraction could not evoke either immediate or delayed-type skin reactions. These studies indicate that the lipopolysaccharide fraction is specific for circulating antibodies and the protein fraction for both circulating antibodies and cell-mediated reactions.

Modification of infection and immune response in the immunized animals.

The active immunization with soluble extracts, led to the development of increased levels of both antibody-mediated and cell-mediated responses simultaneously. Since the sera from these animals showed *in vitro* toxicity on the test fungus it is interesting to study the course of infection and immune response in these animals.

The immunized animals (having a titer of precipitin of 1:16 and a delayed-type of hypersensitive reaction of 14-15 mm) were infected with *T. mentagrophytes* and the course of infection and immune response were examined. The results are shown in Table 8. The complete clinical cure was noticed by 21 days.

The lesions were highly erythematic with increased levels of delayed hypersensitive reactions. There was no increase in the levels of precipitins, but the titer of complement-fixing antibodies was reduced. These studies indicate that active immunization would help in the development of resistance to the dermatomycotic infections also.

TABLE 8. MODIFICATION OF INFECTIONS AND IMMUNE RESPONSES IN ACTIVELY IMMUNIZED ANIMALS.

Antibody types	Early infective phase	Peak of the infection	Declining phase	Clinical recovery
	7 d	14 d	18 d	21 d
Precipitin	1:8	1:8	1:8	1:8
Complement-fixing	1:10	1:4	1:4	1:2
Delayed-type of hypersensitive reactions (induration in mm)	14-15	14	18	18
Duration of the control infection	7 d	21 d	25 d	40 d

4c. Adoptive transfer of cell-mediated immunity to non-sensitized animals.

With the exploration of cellular and biochemical aspects of immune mechanisms, the role of "transfer factor" in transferring the immunity in a number of diseases is extensively studied and recognized. Our studies have shown that active immunization would help in resisting the infection by triggering the immune-cells. Hence the sensitized lymphocytes from the actively immunized animals were examined for their "transfer" capacity.

The lymphocytes from the spleens of sensitized guinea pigs were harvested and washed aseptically and suspended at a cell density of 90×10^6 cells per ml in phosphate buffered saline. Either the whole cells or cell-free extract (prepared by freezing and thawing for 10 times) were administered subcutaneously into the shaved flanks of non-sensitized guinea pigs. After an incubation period of 20 hr, the animals were challenged intradermally with 0.1 ml of soluble extract or protein fraction or lipopolysaccharide fraction of T. mentagrophytes. The cross reactivity with the related dermatophytes was also examined by challenging with the soluble extract or protein fraction or lipo-polysaccharide fraction of T. mentagrophytes. The cross reactivity with the related dermatophytes was also examined by challenging with the soluble extract from Trichophyton violaceum, Trichophyton rubrum and Microsporum canis. The delayed type of hypersensitive reactions developing 24 hr later were recorded (Table 9).

TABLE 9. ADOPTIVE TRANSFER OF CELL-MEDIATED IMMUNITY TO *TRICHOPHYTON MENTAGROPHYTES* IN GUINEA PIGS.

Antigenic material	Skin reaction (in mm) in animals infected with	
	Lymphocytes	Lymphocyte-extract
Trichophyton mentagrophytes soluble extract (20 μg protein)	12	11
Protein fraction	11	10
Lipopolysaccharide fraction (20 μg hexoses)	0	0
Trichophyton violaceum soluble extract (20 μg protein)	10	Not tested
Trichophyton rubrum soluble extract (20 μg protein)	6	Not tested
Microsporum canis (20 μg protein)	9	Not tested

A positive reaction was elicited in the animals injected with both whole cells and their extract. Both the soluble extract and protein fraction but not the lipopolysaccharide fraction evoked delayed hypersensitive reactions. Significant levels of positive reactions with the soluble extracts from other related dermatophytes indicated the cross reactivity.

These studies reveal that lymphocytes sensitized to a dermatophyte do possess a "transfer factor" in addition to various other cellular reactions as observed in some of the systemic mycotic infections like histoplasmosis and coccidioidomycosis. The chemical nature of this "transfer factor" and its in vivo efficacy in curing experimental dermatomycoses are being investigated.

C5. BIOCHEMICAL STUDIES IN *MICROSPORUM CANIS* SPORE GERMINATION AND MECHANISM OF CYCLOHEXIMIDE RESISTANCE

The fungi are an important group of organisms forming a distinct group and possess a relatively simple type of basic organization and high degree of specialization. These features with marked plasticity of form in response to environmental conditions make them suitable organisms for morphogenic studies and as such studies on these fungi should contribute to the understanding of eukaryotic biology as a whole.

Dermatophytes, viz., species of Trichophyton, Microsporum and Epidermophyton constitute the group responsible for superficial infections of keratinized tissues like epidermis, hair and nails. Dermatophytes are generally pleomorphic in nature and produce in their life-cycle three types of spores - macroconidia, microconidia and arthrospores. The spores of these fungi are important because of their pathogenicity and involvement in the spread of infections. The biochemical and physiological studies of sporulation and germination in dermatophytes would help in better understanding of the mode of infection, host-parasite relationship and specialized nature of parasitism.

Among the three genera of dermatophytes, Microsporum is less pleomorphic and produces more macroconidia than microconidia. Hence it was chosen as a representative organism for studying differentiation in dermatophytes.

5a. Growth and sporulation of Microsporum canis.

Several species and strains of Microsporum were tested for their ability to sporulate in media which differ with respect to carbon, nitrogen and vitamin

sources. Only a few strains of M. gypseum viz., I44, I30 and one strain of M. canis, HM 382 sporulated well in Sabouraud's glucose agar (SGA) medium. Alteration of carbon or nitrogen source did not have any significant effect on sporulation of Microsporum spp. However, replacement of peptone in SGA with tryptone and addition of yeast extract and KH_2PO_4 resulted in the increased sporulation in M. canis HM 382, M. gypseum I44 and I30. As the sporulation was quite abundant in M. canis HM 382, compared to other species and strains, it was chosen for morphological and biochemical aspects of spore germination.

M. canis sporulated well at 30°C in about 10-14 days. The optimum pH for sporulation was found to be 6.2. Though decrease in pH did not have any deleterious effect, increase in pH of the medium towards alkalinity resulted in drastic inhibition of growth and sporulation of the organism.

5b. Germination of macroconidia of M. canis.

Distinct morphological changes could be observed by 4 hr when macroconidia were suspended in SG broth. By 6 to 8 hr emergence of germ tubes takes place and by 24 hr more than 95% macroconidia showed multiple germ tubes.

Effect of nutrients: Though 40-45% macroconidia germinate in saline, germ tubes formed by 8 hr failed to elongate and form mycelia. Glucose had no effect on germination either at 30°C or 37°C. On the contrary, peptone at 1% markedly enhanced the germination as in SG broth.

Heat resistance of macroconidia: Macroconidia germinated well at 30°C in peptone broth. Heating the macroconidia for 2 min at 55°C resulted in the loss of ability of macroconidia to germinate both in peptone and SG broth.

Effect of spore concentration : Optimal spore concentration for germination was found to be 3×10^6 macroconidia/ml of germination medium. Increasing the spore concentration to 10×10^6/ml resulted in a decrease in the number of spores germinated by 8 hr.

Effect of peptone concentration: Decreasing the peptone concentration from 1.0% to 0.1% caused 50% inhibition in the germination of macroconidia. At concentration less than 0.1% peptone failed to stimulate germination.

Effect of L-amino acids: As the addition of peptone increased the germination rate of macroconidia, several amino acids were tested for their capacity to stimulate germination. Out of 19 amino acids tested only L-leucine and L-glutamic acid at 10 mM concentration stimulated the germination by 37% and 30% respectively, by 24 hr.

5c. Macromolecular changes.

The macromolecular changes taking place during the active phase of germination (during 4 to 8 hr) and earlier have been examined. The changes in dry weight, protein, nucleic acids, amino acids and nucleotides have been given in Table 1. Despite the increase in the rate of germination dry weight of macroconidia did not show any marked change and so also the protein

TABLE 1. CHANGES IN CHEMICAL COMPOSITION OF *M. CANIS* MACROCONIDIA DURING GERMINATION[a].

	Duration of germination (hr)				
	0	2	4	6	8
Dry weight (mg)[b]	102.25	94.75	110.25	112.0	107.0
Protein (mg)	6.0	5.82	5.62	6.00	5.70
RNA (μg)	1410	1480	1200	1120	1850
DNA (μg)	108	177	152	144	163
Nucleotides (μg)	637	623	492	511	251
Amino acids (mg)	2.32	1.96	1.82	2.10	0.68
Sugar (mg)	2.60	3.0	2.3	2.45	3.35

[a] Germination system contained: Macroconidia, 4×10^6/ml; peptone, 10 mg/ml.

[b] All values are calculated per 10^8 macroconidia.

content. The ribonucleic acid content decreased during early germination and it was followed by considerable increase between 6 to 8 hr of germination. In contrast, the level of nucleotides decreased gradually throughout the 8 hr germination period. Studies using labelled precursors revealed that considerable macromolecular turnover takes place during germination of macroconidia.

5d. Effect of inhibitors on *in vivo* macromolecular synthesis.

Effect of puromycin: Puromycin, which inhibits the protein synthesis by acting as an analogue of amino acyl-tRNA, at 50 and 100 µg/ml failed to inhibit the *in vivo* protein synthesis during germination of macroconidia.

Effect of 2-phenethylalcohol (2-PEA): 2-PEA inhibited the germination and *in vivo* protein synthesis considerably at 10 mM. At concentrations higher than 10 mM, its inhibition was more pronounced.

Effect of cycloheximide: Cycloheximide (CHI), a potent inhibitor of eukaryotic protein synthesis failed to bring about any inhibition of *in vivo* protein synthesis at concentrations ranging from 5 to 250 µg/ml. In contrast, it inhibited the *in vivo* protein synthesis of *Aspergillus niger*, a saprophytic fungus by over 85% at 100 µg/ml. Growth of organism was also not affected even at concentrations of 0.5 to 2.5 mg/ml, while the growth of *A. niger* was completely inhibited at 0.5 mg/ml (Table 2). In order to elucidate the nature of resistance of *M. canis* to CHI, the effect was examined in greater detail.

TABLE 2. EFFECT OF CYCLOHEXIMIDE ON GROWTH AND SPORULATION OF *M. CANIS*.

Organism	Cycloheximide (mg/ml)	Growth[a]	Sporulation
M. canis	0.0	++++	+++
	0.1	++++	++++
	0.5	+++	++
	1.0	++	+
	2.5	++	-
A. niger	0.0	++++	++++
	0.5	-	-

[a] After 7 days. Organisms were grown in SG agar plates.

- nil; + negligible; ++ poor; +++ fair; ++++ good.

The ineffectiveness of CHI on growth and protein synthesis of macroconidia during germination could be due to: a) lack of permeability of the drug into the cell, b) alteration of ribosomes of the organisms, c) detoxification of the drug, and d) adaptation of the organism to CHI.

Lack of permeability of the drug: Alteration in membrane structure during germination of fungal spores has been reported. As a result of such alteration in membrane structure some antifungal agents like pentachlorophenol, respiratory inhibitors can gain access into the spores during germination and become effective. The fact that CHI remained ineffective not only during germination but also in mycelia suggesting that permeability may not be a barrier for the entry of the drug into the macroconidia. Attempts made to permeabilize the macroconidia to CHI by the use of detergents like sodium dodecylsulfate and miconazole nitrate, an antifungal agent were unsuccessful.

Alteration of ribosomes of the organism: As the surface active agents themselves inhibited germination and growth of macroconidia, it was considered necessary to study the effect of CHI on the in vitro protein synthesis of the organism in order to elucidate the nature of resistance of the organism.

5e. Characteristics of in vitro protein synthesis in mycelia and macroconidia.

The S-30 extracts derived from germinating macroconidia and mycelia of M. canis synthesized poly-L-

phenylalanine at 30°C. Unlike in the mycelial extracts, preincubation of macroconidial extracts for 10 min at 30°C decreased the incorporation by 30%. The protein synthetic activity of the S-30 extracts was quite unstable; 50% activity was lost on keeping the extracts at -20°C for 24 hr.

The important characteristics of amino acid incorporation were studied by deletion of essential components or addition of specific inhibitors to the reaction mixture. [The complete reaction mixture contained the following (in μmoles unless otherwise specified) in a total volume of 1.0 ml: Tris-HCl (pH 8.0), 50; magnesium acetate, 10; potassium chloride, 2.5; ammonium chloride, 50; β-mercaptoethanol, 5; spermidine, 2; yeast tRNA, 100 μg; adenosine triphosphate (ATP), 2.5; guanosine triphosphate (GTP), 0.2; phosphoenolpyruvate, 5.0; pyruvate kinase, 10 μg; polyuridylic acid (poly-U), 100 μg; ^{14}C-L-phenylalanine (sp.act. 153-180 mCi/mmole), 0.2 μCi; each of the 19 other amino acids, 0.04; S-30 extract].

The incorporation was dependent on ATP, an energy source, magnesium and poly-U. Incorporation was enhanced by yeast tRNA and to a lesser extent by GTP and pyruvate kinase. The system was sensitive to ribonuclease, puromycin, sodium fluoride, miconazole nitrate and less sensitive to CHI, deoxyribonuclease, chloramphenicol and 2-phenethyl alcohol (Table 3). The protein synthesis was maximal at 25°C and decreased considerably at 37°C and 42°C.

TABLE 3. EFFECT OF INHIBITORS ON THE *IN VITRO* PROTEIN SYNTHESIS IN MACROCONIDIA AND MYCELIA OF *M. CANIS*.

Inhibitors	Macroconidia		Mycelia	
	Incorporation*	Inhibition (%)	Incorporation	Inhibition (%)
Complete system	3608	-	3140	-
+Cyclohexi-mide 50 µg	2445	32	-	-
+Cyclohexi-mide 100 µg	2450	32	2102	34
+Puromycin 100 µg	1542	57	499	85
+2-Phenethyl-alcohol	-	-	2920	8
+Miconazole 50 µg	876	75	644	80
+Chloramphenicol 50 µg	3750	-	3123	1
+RNase 50 µg	292	89	213	94
+NaF 50 µg	1304	64	-	-

* Incorporation expressed as CPM/mg protein.

5f. Effect of CHI and its analogues on *in vitro* protein synthesis.

CHI, at concentrations ranging from 10^{-7}M to 10^{-3}M failed to inhibit *in vitro* protein synthesis both in mycelia and macroconidia. Its analogues like CHI oxime, CHI acetate, isocycloheximide and streptovitacin A were also found to be ineffective. One of the analogues, CHI semicarbazone showed a slight stimulation of the amino acid incorporation.

In contrast to the effects observed in *M. canis*, CHI inhibited the *in vitro* protein synthesis in *Saccharomyces cerevisiae* by 50% at 10^{-4}M and 80% at 10^{-3}M.

The effect of CHI was examined in a heterologous system wherein ribosomes and S-100 fraction of *M. canis* and *S. cerevisiae* were cross-mixed (Table 4). CHI showed over 65% inhibition in the system where the ribosomes are from *S. cerevisiae* and S-100 fraction from *M. canis*. No inhibition was observed when ribosomes of *M. canis* and S-100 fraction of *S. cerevisiae* were used. The results suggest that resistance to CHI in *M. canis* is at the level of ribosomes.

5g. 'Limiting factor' in the macroconidia for protein synthesis.

The fact that germinating conidia have a high incorporating activity as compared to ungerminated conidia, suggest that ungerminated conidia have a limiting factor in protein synthesizing mechanism. Such limiting factor could be: tRNA, amino-acyl tRNA synthetases, transfer enzyme (soluble fraction) and/or

TABLE 4. ^{14}C-PHENYLALANINE INCORPORATION WITH RIBOSOMES FROM MACROCONIDIA OF M. CANIS AND VEGETATIVE CELLS OF S. CEREVISIAE.

Microsporum canis		S. cerevisiae		Cyclohexamide concentration(M)	Inhibition (%)
Ribosomes	S-100	Ribosomes	S-100		
+	+	-	-	0	0
+	+	-	-	10^{-5}	+25
+	+	-	-	10^{-3}	2
-	-	+	+	10^{-4}	50
-	-	+	+	10^{-3}	80
+	-	-	+	10^{-4}	18
-	+	+	-	10^{-4}	58

The incubation system was as described in the text, with 300 μg of S-100 protein and 250 μg of ribosomal protein. S-100 and ribosomes were mixed separately and added to the reaction mixture.

ribosomes. Studies carried out by cross-mixing the ribosomes and S-100 fractions of ungerminated and germinated conidia indicate that ribosomes of ungerminated conidia and not the S-100 fraction that is defective (Table 5). Further, the phenylalanine-tRNA synthetase activities were found to be quite similar in both ungerminated and germinated conidia. The defect in the ribosomes could be in the absence of certain essential proteins or presence of an inhibitor or association of ribonuclease activities with the ribosomes. In fact, our studies indicate that ribosomes of ungerminated conidia have an increased RNase activity compared to ribosomes from germinating conidia.

TABLE 5. ^{14}C-PHENYLALANINE INCORPORATION WITH RIBOSOMES FROM UNGERMINATED AND GERMINATED MACROCONIDIA OF M. CANIS.

Ungerminated		Germinated		^{14}C-phenyl-alanine incorporation*
Ribosomes	S-100	Ribosomes	S-100	
-	-	+	+	3830
+	+	-	-	858
-	+	+	-	5130
+	-	-	+	884

* Incorporation expressed as CPM/mg ribosomal protein.

The incubation system was described in text. 300 µg S-100 protein and 250 µg of ribosomal proteins were used.

C.6. BIOSYNTHETIC POTENTIALITIES OF *CANDIDA* SPECIES*

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* Contributions of Dr. T.K. Narayanan and Dr. V.P. Choudary, research scholars associated with Principal Investigators.

Yeasts, in comparison to other microorganisms, have been more intimately associated with the progress and well being of human race. Their contribution to man's progress has been based largely on: their capacity to bring about rapid and efficient conversion of sugars to alcohols and CO_2; their role in elucidating basic biochemical and metabolic processes in living cell and their varied biochemical potentialities in producing several metabolic products.

6a. Production and mode of action of aromatic alcohols.

With the exception of a few reports, the biosynthetic ability of Candida species to produce aromatic alcohols has not received a thorough examination. Hence these species have been examined for their ability to produce various compounds when grown on L-phenylalanine, L-tyrosine, and L-tryptophan. A simple method of extraction and purification of these compounds from culture filtrates has been developed. Besides alcohols the presence of other compounds, intermediates in culture filtrates, has also been tested. The production of alcohols and hydroxy acids by Candida species has been quantitated and the use of Candida species to produce labelled compounds investigated. The results revealed the biosynthetic ability of Candida spp. to produce β-phenethylalcohol and β-phenyllactic acid (pages, 80-87); β-(4-hydroxyphenyl)-ethanol and β-(4-hydroxyphenyl)lactic acid (pages, 88-93) and β-indole ethanol and β-indole lactic acid (pages,94-99).

Extensive literature is available on the mode of action of PEA in mammalian and plant cells, bacteria and viruses. The isolation of PEA, HOPEA and IEA has

provided an opportunity to make a comparative study of their mode of action in yeasts. Their effect on dimorphism, growth inhibition and reversibility, respiration, uptake of labelled precursors, macromolecular synthesis and membrane function in C. albicans, has been investigated and discussed. The results (details presented in the Annual Report 1975-76, pages 60-89) suggest that these alcohols interfere with several cellular processes, and their effect can be explained by their interaction with cell membrane.

6b. Enzymology of PEA biosynthesis in Candida guilliermondii

Though the pathways involved in the biosynthesis of phenylalanine, tyrosine and tryptophan in yeasts have been extensively studied, little is known about the enzymology of their catabolism. The enzymology of the biosynthesis and regulatory aspects of PEA and IEA are of great interest in view of their reported auto-antibiotic activity. Hence in the present study, investigations have been carried out on the enzymology and regulation of the biosynthesis of PEA and PLA from L-phenylalanine in Candida guillermondii (pages 100-105).

PRODUCTION OF 2-PHENETHYL ALCOHOL AND 2-PHENYLLACTIC ACID IN CANDIDA SPECIES

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SUMMARY: 2-Phenethyl alcohol (2-PEA) and 2-phenyllactic acid (2-PLA) were isolated from the culture filtrates of Candida species grown in media containing peptone or phenylalanine as nitrogen source. These compounds were characterized by comparing their UV, IR, and NMR spectral properties with authentic samples. Candida species differed markedly in their production of 2-PEA and 2-PLA. Experiments using [^{14}C] -phenylalanine indicated that both 2-PEA and 2-PLA are synthesised from L-phenylalanine. A pathway for the biosynthesis of 2-PEA from L-phenylalanine has been proposed.

INTRODUCTION

The biosynthetic ability of species of Saccharomyces and Candida have been extensively exploited in the production of alcohols (1), vitamins (2) and enzymes (3). 2-Phenethyl alcohol (2-PEA) was isolated from culture filtrates of Candida albicans when grown in Sabouraud's broth (4). Whether the production of this compound is unique to only C. albicans or shared by other species of the genus Candida, as well its physiological role and the enzymology involved in its biosynthesis are some of the aspects about which nothing is known. The purpose of this study is to examine the ability of both pathogenic and nonpathogenic species of Candida to produce 2-PEA and the intermediates involved in its biosynthesis.

MATERIALS AND METHODS

C. albicans Z248, C. guilliermondii Z55, C. krusei Z70 and C. tropicalis Z56 were obtained from London School of Hygiene and Tropical Medicine, London and C. intermedia from V. P. Chest Institute, New Delhi, India. Stock cultures are maintained on Sabouraud's glucose agar.

Erlenmeyer flasks (500 ml) each containing 200 ml of the medium were inoculated with 5 ml of 24 hr broth culture in the same medium. The cells were grown at 31 or 37 C on a rotary shaker for 2 or 7 days. The cells were sedimented by centrifugation at 2000g for 20 min in a Sorvall RC-2B and the supernatant (designated as culture filtrate) was decanted. The cell pellet

81

Table 1. Physicochemical and spectral properties of 2-PEA and 2-PLA isolated from culture filtrates of Candida species.

Property	2-PEA*	2-PLA**
Liquid/solid	oil (colorless)	leaflet crystals
m.p./b.p.	220 C	120 C
Solubility in acetone	highly soluble	highly soluble
ether	"	"
chloroform	"	slightly soluble
Optical rotation	-	+9°
Rf values	0.72 (TLC)[e]	0.96 (paper chromatography)[f]
U.V. (λ max)[a]	259 nm	258 nm
I.R. absorption[b] range Cm^{-1}	3575 (w)) C_1-OH 3350 (b))	3450 (sh) C_2-OH 1740 (sh) C_1=O
	1460 (sh)) 1508 (sh)) C_6H_5- 1615 (sh))	1460 (sh)) 1500 (sh)) C_6H_5- 1610 (sh))
NMR signals[c] (δ values)	7.40 singlet C_6H_5-	7.40 singlet C_6H_5-
	2.90 triplet $-CH_2$-	4.30 quadruplet HC
	3.90 triplet $-CH_2$-	3.0 quadruplet $-CH_2$-
	1.70 singlet C -OH	2.60 multiplet DMSO
On exchange with D_2O[d]	singlet at δ 1.70 of C_1-OH disappeared	

* From C. guilliermondii ** From C. tropicalis

a Ethyl alcoholic solution (10 mg/100 ml), Unicam SP 700A

b Neat's spectrum (2-PEA) and Nujol spectrum (2-PLA), Carl Zeiss Model W10

c 2-PEA in $CDCl_3$ and 2-PLA in DMSO-D_6 (10-15%), Varian Model T60 MH_2

d 2-PEA was treated with D_2O and kept overnight for exchange

e Solvent system: petroleum ether:ether (1:5)— Identification by iodination

f Solvent system: benzene:acetic acid:water (15:6:3) — Identification by spraying with 0.1% $KMnO_4$

(sh) sharp; (b) broad; (w) wide.

was washed twice with distilled water and the washings were pooled with culture filtrate.

Extraction and purification: Procedure 1 —The filtrate from 2 litres of culture was extracted with chloroform (1 vol for every 10 vol) and the extraction was repeated four times. The chloroform extract was dried over

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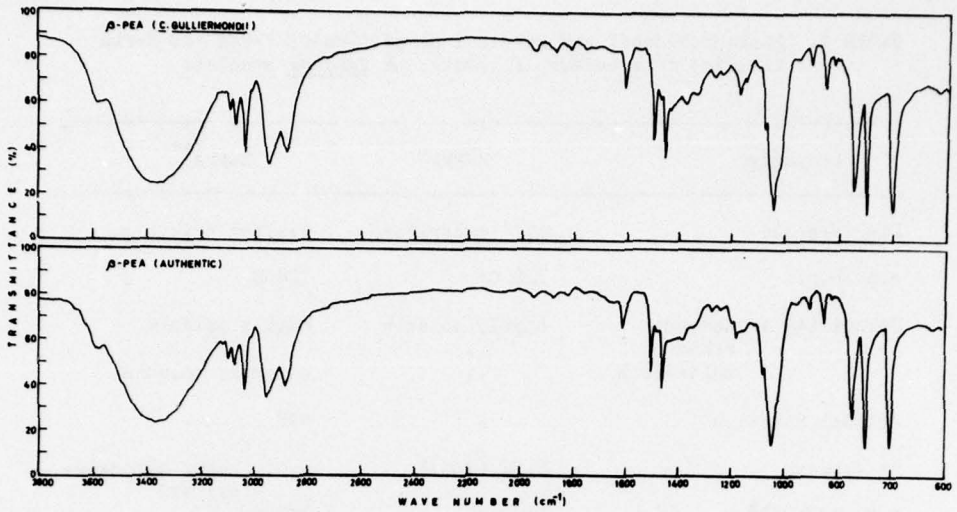

FIG. I. INFRARED SPECTRA OF THE ISOLATED AND AUTHENTIC β-PEA

anhydrous sodium sulphate and evaporated to dryness at 37 C. The oily material thus obtained was treated with 5 ml of acetone and the acetone insoluble material was filtered off. The filtrate was dried and the acetone precipitation was repeated twice. The acetone-free material (250 mg) was applied to 50 X 1.5 cm column of silica gel and eluted with petroleum ether : ether (4:1 v/v) mixture. The first 100-150 ml of the eluate was evaporated at 37 C and the residue (30 mg) was dried in vacuum and used for spectral studies.

Procedure 2 —Step A: One litre of the culture filtrate was extracted with solvent ether (2 vol for every 10 vol) twice and the ether extract was dried at 37 C. The material was taken in 100-150 ml of warm water and the pH was adjusted to 8.0 with sodium bicarbonate and extracted twice with ether. The ether extract was dried over anhydrous sodium sulphate and evaporated at 37 C. The oily material was treated with 5 ml of acetone and the acetone-insoluble material was filtered off and the filtrate was evaporated at 37 C and dried in vacuum and used for spectral analysis. Step B: The alkaline aqueous solution from step A was acidified to pH 3.0 with HCl and the process of ether extraction and acetone treatment was repeated as in step A. The crystalline material obtained was dried in vacuum and used for spectral studies.

RESULTS AND DISCUSSION

From the culture filtrates of C. guilliermondii grown in glucose-peptone broth (Table 2, medium 1) an oily material was isolated by a modified extraction and purification procedure 1 (4). Its physicochemical and UV, IR (Fig.1) and NMR (Figs. 2, 3) spectral properties have been examined (Table 1). This compound was characterised as 2-PEA and confirmed with authentic 2-PEA (BDH sample). Besides C. guilliermondii, other species of Candida like C. albicans,

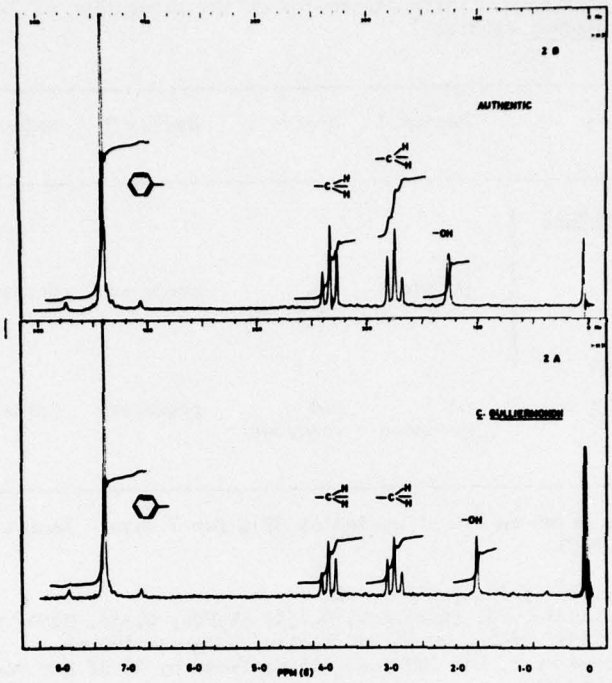

FIG. 2. NUCLEAR MAGNETIC RESONANCE SPECTRA OF ISOLATED (2A) AND AUTHENTIC (2 B) β-PEA

C. krusei and C. intermedia also produced 2-PEA under identical conditions (Table 2). However, only in C. tropicalis no detectable amount was found.

To define the culture medium, peptone was replaced by ammonium sulphate at a concentration of 0.25% (Table 2, medium 2). At this concentration ammonium sulphate supports good growth of all species of Candida* but none of the species produced 2-PEA. This indicated that amino acid(s) present in peptone is required for the biosynthesis of 2-PEA. Hence, the effect of L-phenylalanine, a compound possibly utilized in the biosynthesis of 2-PEA was examined. With L-phenylalanine as nitrogen source all species including C. tropicalis produced 2-PEA (medium 3). The presence of ammonium sulphate along with L-phenylalanine, however, had not interfered in the biosynthesis of 2-PEA (medium 4).

The possible presence of other compounds in culture filtrates was examined by altering the method of extraction (procedure 2). By this procedure, a

* V. P. Chowdary and G. Ramananda Rao, unpublished data.

89

Table 2. Influence of nitrogen source on the production of 2-PEA by Candida species *

Species	Medium 1	Medium 2	Medium 3	Medium 4
C. guilliermondii	produced	not produced	produced	produced
C. albicans				
C. krusei				
C. intermedia				
C. tropicalis	not produced	not produced	produced	traces

* Cells were grown in 2 L of medium at 37 C for 7 days. Isolation was by Procedure 1.

Medium 1 : glucose, 4%; peptone, 2%.
Medium 2 : glucose, 2%; $(NH_4)2SO_4$, 0.25%; KH_2PO_4, 0.35%; $MgSO_4.7H_2O$, 0.25%; $CaCl_2$, 0.25% and biotin, 3 ug per 100 ml.
Medium 3 : same as 2, but $(NH_4)_2SO_4$ is replaced by 1% of L-phenylalanine (Sigma)
Medium 4 : both $(NH_4)_2SO_4$ and L-phenylalanine were present at the same concentrations as in media 2 and 3.

Table 3. Production of2-PEA and 2-PLA by Candida species*

Species	2-PEA (mg)	2-PLA (mg)	Total of 2-PEA & 2-PLA (mg)	Conversion
C. guilliermondii	578	87	665	66.5%
C. krusei	308	28	336	33.6%
C. intermedia	158	378	536	53.6%
C. albicans	168	412	580	58 %
C. tropicalis	18	620	638	63.8%

* Cells were grown in 1 L of medium 3 at 31 C for 2 days. Isolation was by procedure 2.

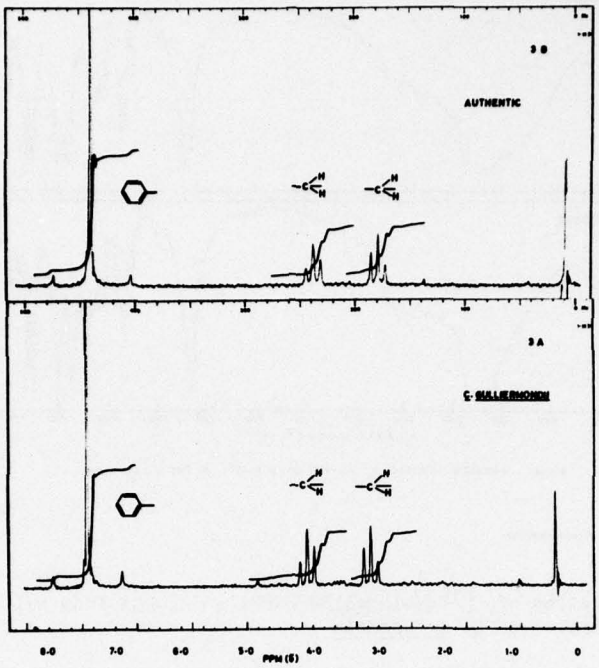

FIG. 3. NUCLEAR MAGNETIC RESONANCE SPECTRA OF ISOLATED (3A) AND AUTHENTIC (3B) β-PEA AFTER D_2O EXCHANGE

crystalline material in high yields was isolated from C. tropicalis and was characterised as 2-phenyllactic acid (2-PLA) by its physicochemical, IR (Fig. 4) and NMR (Fig. 5) spectral properties (Table 1) and it was confirmed with authentic sample of 2-PLA (Sigma).

Employing procedure 2, the production of 2-PEA and 2-PLA in Candida species has been quantitated (Table 3). Candida species differed markedly in their capacity to produce these compounds. C. guilliermondii had produced high amounts of 2-PEA and low amounts of 2-PLA. The same is true with C. krusei while the reverse is the case with C. tropicalis. The conversion of L-phenylalanine into 2-PEA and 2-PLA (together) by various species ranged from 34 to 67%.

Experiments using [^{14}C](U)-phenylalanine were also carried out. Labelled 2-PEA and 2-PLA were isolated from culture filtrates of C. intermedia (Table 4). The total yield of both labelled 2-PEA and 2-PLA was about 52%. This agreed

86

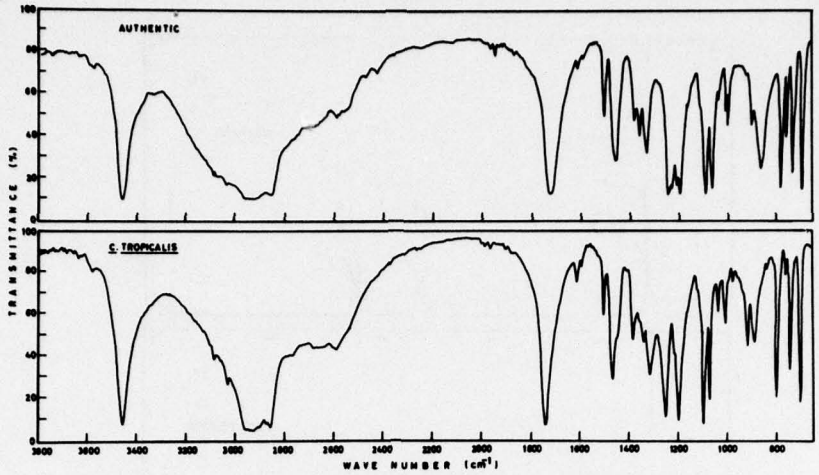

FIG. 4. INFRARED SPECTRA OF ISOLATED AND AUTHENTIC β-PHENYLLACTIC ACID

Table 4. Isolation of [^{14}C]-labelled 2-PEA and 2-PLA from culture filtrates of C. intermedia

Phenylalanine left in the culture filtrate after extraction			1.2×10^6 cpm
Phenylalanine utilized			9.8×10^6 cpm
Counts in 2-PEA (190 mg)	1.6×10^6 cpm		5.1×10^6 cpm
Counts in 2-PLA (410 mg)	3.5×10^6 cpm		
% Incorporation into 2-PEA	16.3		52.3
% Incorporation into 2-PLA	36.0		

Cells were grown in medium 3 supplemented with 8 uCi of [^{14}C]-(U)-L-phenylalanine (Sp. activity, 153 mCi/mmole, obtained from BARC, Bombay, India)

well with the quantitative data obtained in experiment using cold L-phenylalanine (Table 3).

The data presented reveal that all the five species of Candida are 2-PEA producers and they possess the ability to convert a normal metabolite like L-phenylalanine into 2-PLA and 2-PEA. 2-PEA is possibly biosynthesised by

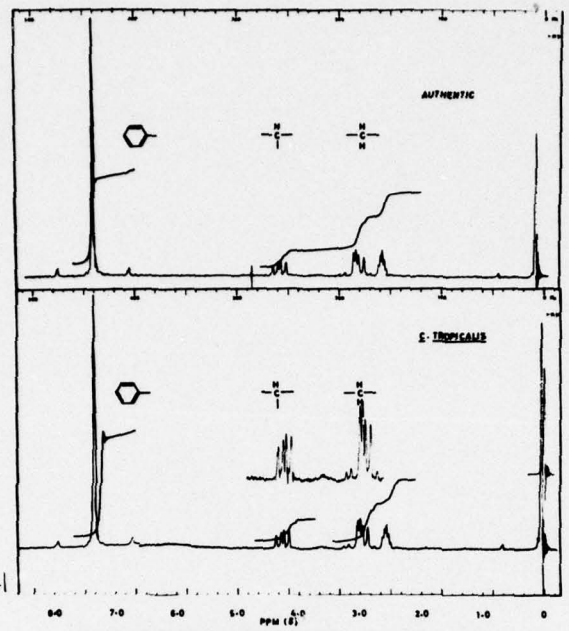

FIG. 5. NUCLEAR MAGNETIC RESONANCE SPECTRA OF ISOLATED AND AUTHENTIC β-PHENYLLACTIC ACID

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the following pathway: L-Phenylalanine — Phenylpyruvic acid — Phenyllactic acid — Phenethyl alcohol.

The presence of amino acid pools in C. utilis (5) and their excretion into the medium during fermentation (6) have been reported. The conversion of excess phenylalanine in the mobile intracellular pool into 2-PEA and its excretion is a possibility.

ACKNOWLEDGEMENTS

We wish to thank Prof. M. Sirsi, Prof. T. R. Kasturi (Department of Organic Chemistry) and Prof. V. S. R. Rao (Molecular Biophysics Unit) for helpful discussions.

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Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species

T. K. Narayanan and G. Ramananda Rao

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Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species[1]

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NARAYANAN, T. K., and G. R. RAO. 1976. Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species. Can. J. Microbiol. **22**: 384–389.

Two crystalline compounds were isolated from the culture filtrates of *Candida* species grown in synthetic medium supplemented with L-tyrosine as the sole source of nitrogen. These compounds were characterized as β-(4-hydroxyphenyl)ethanol (HOPEA) and β-(4-hydroxyphenyl)lactic acid (HOPLA). The production of these compounds in five species (both pathogenic and non-pathogenic) was compared and marked differences were revealed. Experiments using L-[^{14}C]tyrosine indicated that both HOPEA and HOPLA are synthesized from L-tyrosine.

NARAYANAN, T. K., et G. R. RAO. 1976. Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species. Can. J. Microbiol. **22**: 384–389.

On a isolé deux composés cristallins des filtrats de culture d'espèces de *Candida* cultivées dans un milieu synthétique additionné de L-tyrosine comme seule source d'azote. Ces composés ont été caractérisés comme étant l'éthanol-β-(4-hydroxyphényl) (HOPEA) et l'acide-β-(4-hydroxyphényl)lactique (HOPLA). La production de ces composés chez cinq espèces (pathogènes et non-pathogènes) a été comparée et des différences marquées ont été observées. Des expériences utilisant la L-tyrosine [^{14}C] démontrent que l'HOPEA ainsi que l'HOPLA sont synthétisés à partir de la L-tyrosine.

[Traduit par le journal]

Introduction

β-Phenethyl alcohol (β-PEA) production by *Candida albicans* grown in Sabouraud's glucose broth has been reported (4). This compound and hydroxy acid, viz., β-phenyllactic acid (β-PLA), have been isolated from several species of *Candida* grown in a defined medium containing L-phenylalanine (6). When L-phenylalanine is replaced by L-tryptophan, *Candida* species produced β-indoleethanol and β-indolelactic acid.[2]

Production of β-(4-hydroxyphenyl)ethanol-(tyrosol) (HOPEA) by *Saccharomyces cerevisiae* (2, 9) and *Gibberella fugikuroi* (1) and β-(4-hydroxyphenyl)lactic acid (HOPLA) by *Oidium lactis* (3), *Proteus vulgaris*, *Escherichia coli* (7), and *Bacillus subtilis* (8) has been reported earlier. In this paper we are reporting the production and quantitation of both HOPEA and HOPLA by pathogenic and non-pathogenic species of *Candida*.

Materials and Methods

Organisms

Candida albicans Z248, *C. guilliermondii* Z55, *C. krusei* Z70, and *C. tropicalis* Z56 were obtained from London School of Hygiene and Tropical Medicine, London, and *C. intermedia* from V.P. Chest Institute, New Delhi, India. Stock cultures were maintained on Sabouraud's glucose agar.

Medium and Growth Conditions

The following medium was contained in 100 ml of distilled water: glucose, 2 g; KH_2PO_4, 0.35 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; biotin, 3 µg; and L-tyrosine, 0.1 g (Sigma Chemical Co., St. Louis, Missouri, USA). The pH of the medium was 7.0 and it was sterilized by autoclaving at 120 °C for 15 min.

Erlenmeyer flasks (500 ml) each containing 200 ml of the medium were inoculated with 5 ml of a 24-h broth culture prepared in the same medium. The cells were grown at 31 °C on a rotary shaker for 2 days. The cells were sedimented by centrifugation at 2000 *g* in a Sorvall RC2-B centrifuge and the supernatant (designated as culture filtrate) was decanted. The cell pellet was washed twice with distilled water and the washings were pooled with the culture filtrate.

Isolation of Compounds from Culture Filtrates

Step A

One litre of the culture filtrate was extracted twice with 200 ml of ethyl ether each time and the ether extract was evaporated to dryness at 37 °C. The crystalline material was taken up in 100–150 ml of warm water and the pH adjusted to 8 with $NaHCO_3$ and extracted twice with ether. The ether extract was dried over anhydrous Na_2SO_4 and the solvent evaporated at 37 °C. The crystalline material was treated with 5 ml of acetone, the acetone insoluble-material filtered off, and the filtrate evaporated at 37 °C and dried in vacuum (compound I).

[1]Received June 20, 1975.

[2]Presented at the 43rd Annual Meeting of the Society of Biological Chemists (India), held at Ludhiana, India, during November 1974.

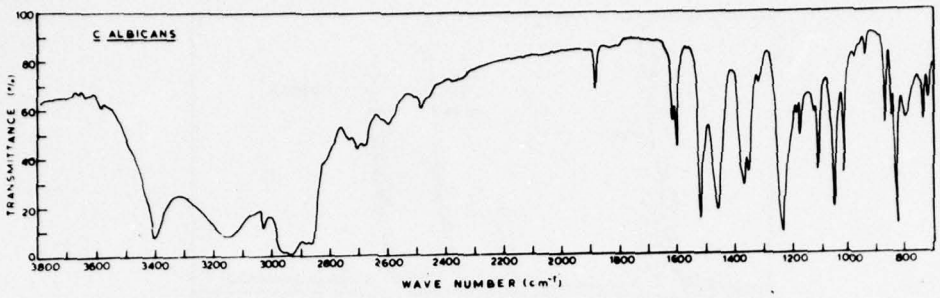

FIG. 1. Infrared spectrum of β-(4-hydroxyphenyl)ethanol isolated from *C. albicans*.

Step B

The alkaline aqueous solution left after ether extraction from Step A was adjusted to pH 3 with HCl and the process of ether extraction and acetone treatment in Step A was repeated. The crystalline material obtained was dried in vacuum (compound II).

Chromatography

Compound I was run on thin-layer chromatography (tlc) on silica gel (National Chemical Laboratory, Poona, India) using a solvent system consisting of ether – petroleum ether (bp 40 °C to 60 °C) 4:1 (v/v). The compound was detected by spraying with $FeCl_3$ solution.

Compound II was chromatographed on Whatman No. 1 paper with a solvent system consisting of benzene – acetic acid – water 5:2:1 (v/v) along with an authentic sample of HOPLA (Sigma Chemical Co., St. Louis, Missouri, USA). Compounds were detected by spraying with $FeCl_3$ solution.

Spectra

The ir spectra (Nujol Mull) of the compounds were determined in Carl Zeiss model W10 spectrophotometer

The nmr spectra of the compounds (10–15% solution) in dimethyl sulfoxide (DMSO)D_6 were determined in a Varian model T_{60} MHz instrument. For compound I the spectrum was also taken after D_2O exchange.

The uv spectra of the compounds (10 mg/100 ml ethanol) were determined in a Unicam Spectrophotometer model SP 700A.

Mass spectrum of compound I was taken in an AEI MS 70z mass spectrometer fitted with electron bombardment source.

Results and Discussion

The ir spectrum of compound I (HOPEA) is shown in Fig. 1. Assignments of various peaks are aromatic ring (phenyl group) at 1460 cm^{-1}, 1520 cm^{-1}, and 1615 cm^{-1}; phenolic hydroxyl group at 3400 cm^{-1}; C_1-hydroxyl group at 3150 cm^{-1} (which is mostly hydrogen-bonded). Peaks at 1240 cm^{-1} and 1050 cm^{-1} further support the presence of hydroxyl groups.

The nmr spectrum of compound I (HOPEA) (Fig. 2) showed a pair of doublets (J = 8 Hz) centered at δ 7 corresponding to $-C_6H_4-$. The triplet (J = 4.5 Hz) at δ 4.5 showed the presence of C_1—OH coupled to methylene protons which disappear on D_2O exchange. The triplet for $-CH_2-$ group at δ 3.6 merged with the singlet of phenolic —OH which disappeared on D_2O exchange and the triplet for $-CH_2-$ group becomes clear. The other triplet (J = 6.4 Hz) at δ 2.8 for $-CH_2-$ merges with the signal for DMSO. The absence of methyl group singlet between δ 1 and δ 2 and the presence of two triplets indicate that $-C_6H_4-$ is separated from C_1—OH by an ethylene linkage. The signal at δ 4.3 is due to HDO.

From the mass spectrum the molecular weight of the compound I (HOPEA) was determined as 138 (Fig. 3).

The phenolic hydroxyl in compound I (HOPEA) was further confirmed by its color reactions: with $FeCl_3$ it gave a blue color and with hot concentrated H_2SO_4 it yielded a red color.

The physicochemical properties of compound I (HOPEA) are shown in Table 1.

The ir spectrum of compound II is shown in Fig. 4. A sharp peak at 3450 cm^{-1} corresponds to the C_2-hydroxyl group; a broad peak at 3200 cm^{-1} to phenolic hydroxyl; and a characteristic peak at 1740 cm^{-1} to the carbonyl group. As compound I, compound II showed peaks at 1460 cm^{-1}, 1520 cm^{-1}, and 1615 cm^{-1} for the phenyl group; peaks at 1100 cm^{-1} and 1250 cm^{-1} further support the presence of hydroxyl groups.

The nmr spectrum of compound II (Fig. 5) showed a pair of doublets (J = 8 Hz) centered at δ 7 for the $-C_6H_4-$ group. The quartet signals centered at δ 4.2 (J = 4 Hz) and δ 2.8 indicated that protons of $-\overset{H}{\underset{|}{C}}-$ and $-\overset{H}{C}-$ are coupled with each other.

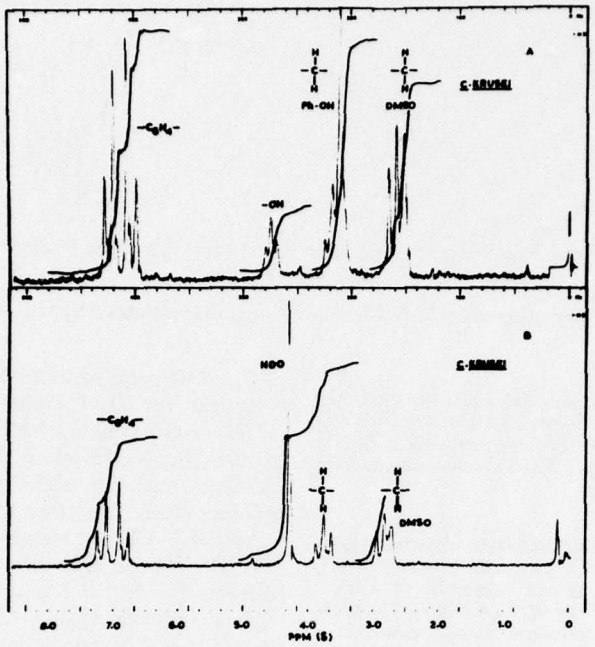

FIG. 2. (A) Nuclear magnetic resonance spectrum of β-(4-hydroxyphenyl)ethanol (isolated from *C. krusei*) in DMSO-D6. (B) Nuclear magnetic resonance spectrum of β-(4-hydroxyphenyl)ethanol (isolated from *C. krusei*) in DMSO-D6 + D_2O.

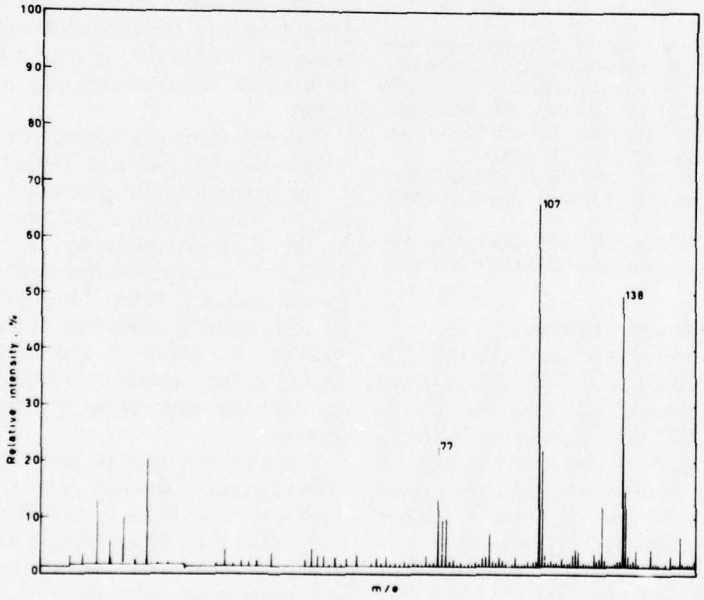

FIG. 3. Mass spectrum of β-(4-hydroxyphenyl)ethanol (isolated from *C. krusei*).

TABLE 1. Physicochemical properties of compound I β-(4-hydroxyphenyl)ethanol and compound II β-(4-hydroxyphenyl)lactic acid

Properties	HOPEA	HOPLA
Solid	Crystalline needle-shaped	Crystalline leaflets
mp	93 °C	168 ± 1 °C
Solubility:		
acetone	Highly soluble	Highly soluble
chloroform	Sparingly soluble	Sparingly soluble
water	Soluble	Soluble
ether	Highly soluble	Highly soluble
Optical rotation	—	+20° at 30 °C (*c* 2.5 in ethanol)
R_F value	0.46*	0.04†
λ_{max} (nm)	275	275

*Thin-layer chromatography on silica gel using a solvent system of ether – petroleum ether (bp 40 °C to 60 °C) 4:1 (v/v); identification by spraying with $FeCl_3$ solution.

†Paper chromatography on Whatman No. 1 using a solvent system of benzene – acetic acid – water, 5:2:1 (v/v); identification by spraying with $FeCl_3$ solution.

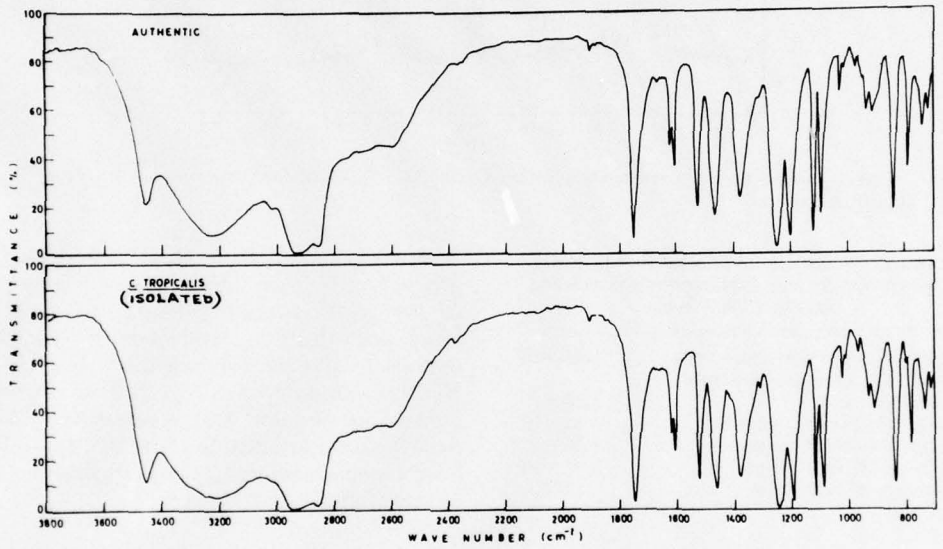

FIG. 4. Infrared spectra of authentic and isolated (*C. tropicalis*) β-(4-hydroxyphenyl)lactic acid.

Based on these spectral properties, compound II is characterized as β-(4-hydroxyphenyl)lactic acid. Its spectra are identical with that of the authentic sample (Sigma Chemical Co., St. Louis, Mo., USA). Its physicochemical properties are shown in Table 1.

Labelled HOPEA and HOPLA were isolated from culture filtrates of *C. intermedia* grown in medium supplemented with L-[U-^{14}C]tyrosine (sp. act. 189 mCi/mmol) (Bhabha Atomic Research Centre, Bombay, India). The total incorporation was 10% (Table 2).

All the five species of *Candida* tested produced both HOPEA and HOPLA. However, they differed considerably in the quantities and proportions they produced (Table 3). *Candida guilliermondii* and *C. krusei* produced more HOPEA than HOPLA; the reverse is true with *C. tropicalis*. *Candida intermedia*, and *C. albicans* produced both compounds in almost equal

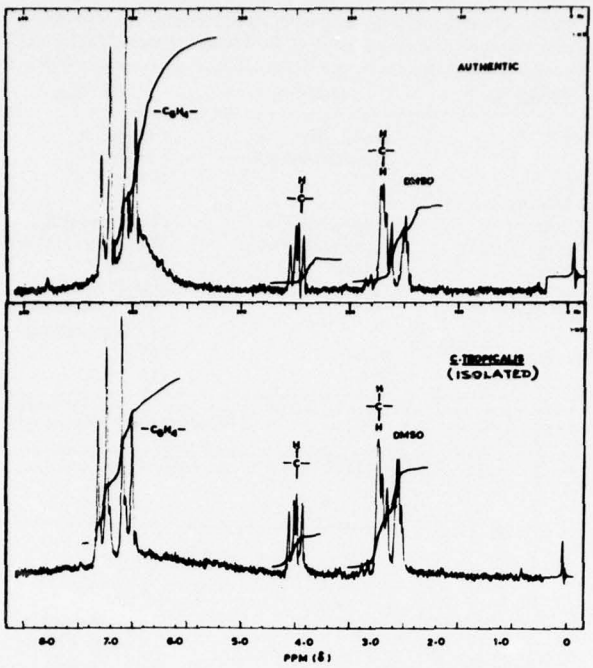

FIG. 5. Nuclear magnetic resonance spectra of authentic and isolated (*C. tropicalis*) β-(4-hydroxyphenyl)lactic acid.

TABLE 2. Production of labelled β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *C. intermedia*

Counts of L-[U-^{14}C]tyrosine added	3.77×10^7
Counts left in the culture filtrate after extraction	1.15×10^7
Counts taken up by the cells	2.62×10^7
Counts in HOPEA (73 mg)	1.97×10^6
Counts in HOPLA (41 mg)	0.7×10^6
Percentage incorporation	10%

amounts. The pattern of HOPEA and HOPLA production by each organism is similar to that of β-phenethylalcohol (β-PEA) and β-phenyllactic acid (β-PLA) production by the same organisms. But the total yield of HOPEA and HOPLA ranged from 10 to 21% of L-tyrosine used in the medium. This is considerably lower than the total production of β-PEA and β-PLA from L-phenylalanine by these organisms which ranged from 34 to 67% (6).

TABLE 3. Production of β-(4-hydroxyphenyl)ethanol and β-(4-hydroxyphenyl)lactic acid by *Candida* species

Yeasts	Yield in mg/litre of medium containing 1 g of L-tyrosine			% of L-tyrosine conversion to HOPEA and HOPLA
	HOPEA	HOPLA	Total	
C. guilliermondii	160	51	211	21.1
C. krusei	120	20	140	14.0
C. intermedia	63	45	108	10.8
C. albicans	51	47	98	9.8
C. tropicalis	11	107	118	11.8

The autoinhibitory effect of β-PEA on the growth of these *Candida* species and differences in their susceptibility to β-PEA have been reported (5). The reasons for the lower production of HOPEA and HOPLA are not known. However, HOPEA could exert higher autoinhibitory effect than β-PEA, thus regulating its own biosynthesis. Alternatively, a low intracellular level of L-tyrosine either due to low uptake or rapid *in situ* utilization through other metabolic pathways may be responsible.

The data presented reveal the ability of *Candida* species to convert a normal metabolite such as L-tyrosine to HOPEA and HOPLA. They are possibly biosynthesized by the following pathways:

L-tyrosine → hydroxyphenyl pyruvic acid → hydroxyphenyllactic acid

↓

hydroxyphenylacetaldehyde → hydroxyphenylethanol.

Acknowledgments

We thank Prof. M. Sirsi, T.R. Kasturi (Department of Organic Chemistry), and Prof. V.S.R. Rao (Molecular Biophysics Unit) for helpful discussions. One of us (GRR) is grateful for the financial support received from the office of Naval Research, Washington, D.C., under contract No. N00014-71-C-0349, for his research program.

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Beta-Indoleethanol and Beta-Indolelactic Acid Production by *Candida* Species: Their Antibacterial and Autoantibiotic Action

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Candida spp. grown in synthetic medium supplemented with L-tryptophan as sole nitrogen source produced β-indoleethanol (β-IEA) and β-indolelactic acid (β-ILA). These compounds isolated from the culture filtrates were characterized by ultraviolet, infrared, and nuclear magnetic resonance spectral studies. Using DL-[^{3}H]trypotophan in the medium, labeled β-IEA and β-ILA were isolated. Further, β-IEA was produced as a result incubating log-phase cells of *C. albicans* with β-ILA. Both β-IEA and β-ILA inhibited the growth of gram-positive and -negative bacteria. Autoantibiotic action of these compounds on *Candida* spp. and the reversal of this inhibition were studied.

Candida spp. produced β-phenethylalcohol (5, 7) and its corresponding hydroxy acid, β-phenyllactic acid (7). The autoantibiotic action of β-phenethylalcohol has also been reported (5, 6). Production of β-indoleethanol (tryptophol; β-IEA) and β-indolelactic acid (β-ILA) by *Agrobacterium tumefaciens* (3), *Diplodia natalensis* (1), *Acetobacter xylinum* (4), *Aspergillus niger* (2), and *Rhizobium* spp. (8, 9) has been described. In this paper we report the production and quantitation of β-IEA and β-ILA by both pathogenic and nonpathogenic species of *Candida* grown in the presence of L-tryptophan as the sole source of nitrogen. These compounds have also been examined for their antibacterial and autoantibiotic action.

MATERIALS AND METHODS

Organisms. *Candida albicans* Z248, *C. guilliermondii* Z55, *C. krusei* Z70, and *C. tropicalis* Z56 were obtained from the London School of Hygiene and Tropical Medicine, London, and *C. intermedia* was obtained from the V. P. Chest Institute, New Delhi, India. Stock cultures were maintained on Sabouraud glucose agar. *Escherichia coli, Proteus vulgaris, Paracolobactrum aerogenoides, Aerobacter aerogenes, Bacillus subtilis, B. megaterium,* and *B. cereus* were from the culture collection of this laboratory and were maintained on nutrient agar slants.

Isolation of compounds. The growth of organisms and the procedure for the isolation of compounds and their purification were similar to those described earlier (7).

Spectra. The infrared spectra were taken in Nujol in a Carl Zeiss model W10 spectrophotometer and the nuclear magnetic resonance spectra of the compounds (10 to 15%) were taken in a Varian model T60MHz. The ultraviolet spectra of the compounds in aqueous solution (10 mg/100 ml) were obtained in a Unicam SP 700A recording spectrophotometer.

Reversibility of growth inhibition caused by β-IEA and β-ILA. Side-arm Erlenmeyer flasks (250 ml), each containing 100 ml of medium (described in Table 5), were inoculated with 1 ml of 24-h broth culture in the same medium. After 8 h of incubation on a rotary shaker (200 rpm) at 30 C, the compounds were added aseptically to two flasks and incubated further for another 8 h. The cells thus exposed to the compounds were centrifuged and washed four times with sterile medium, suspended in the same volume of fresh medium, and incubated. The culture filtrate from the flask to which β-ILA was added was extracted using the isolation procedure previously described, and the product was identified by chromatography.

RESULTS AND DISCUSSION

The physicochemical properties of β-IEA and β-ILA are shown in Table 1.

The infrared spectrum of β-IEA is shown in Fig. 1. The assignments of the peaks are as follows: (i) a sharp peak at 3,400 cm^{-1} corresponds to of indole nucleus; (ii) a broad peak at 3,200 cm^{-1} corresponds to C_1—OH, which is mostly hydrogen bonded; (iii) peaks at 1,430, 1,470, and 1,620 cm^{-1} show that it is an aromatic compound; (iv) peaks at 1,050, 1,100, and 1,190 cm^{-1} further support the presence of the —OH group.

The infrared spectrum of β-ILA is shown in Fig. 2. The assignments of the various peaks are as follows: (i) a sharp peak at 3,400 cm^{-1} corresponds to ; (ii) a peak at 3,500 cm^{-1}

TABLE 1. *Physicochemical properties of β-IEA and β-ILA isolated from a culture filtrate of Candida species*

Property	β-IEA	β-ILA
Liquid/solid	Crystalline leaflet	Microcrystalline powder
Odor	Fecal odor	Fecal ordor
Melting point (C)	58–59	100 ± 1
Solubility in:		
Acetone	Highly soluble	Highly soluble
Chloroform	Soluble	Sparingly soluble
Ether	Soluble	Soluble
Water	Sparingly soluble	Soluble
Ultraviolet spectrum (λ_{max}) (nm)	278	278
Optical rotation		+6° at 30 C (ca. 2.5 in ethanol)
R_f value	0.41[a]	0.40[b]

[a] Thin-layer chromatography solvent system: petroleum ether (40 to 60 C)-ether (15:25, vol/vol); identification by iodination.

[b] Paper chromatography solvent system: benzene-acetic acid-water (15:6:3, vol/vol/vol); identification by spraying with 0.1% $KMnO_4$.

corresponds to C_2—OH; (iii) sharp peaks at 1,700 and 1,720 cm^{-1} correspond to $\diagdown C{=}O$; (iv) peaks at 1,400, 1,480, and 1,640 cm^{-1} show the aromatic nature of the compound; (v) peaks at 1,080, 1,100, and 1,200 cm^{-1} further support the presence of the —OH group.

The nuclear magnetic resonance spectra of β-IEA are shown in Fig. 3 and 4. A multiplet centered at 7.4δ corresponds to the indole nucleus. The triplets at 3.9δ (J 6Hz) and 2.9δ (J 6Hz) correspond to two $-CH_2-$ groups coupled to each other. A sharp singlet at 2δ and a broad singlet at 8.2δ correspond to C_1—OH and $\diagdown N \diagup H$ of indole, respectively, which disappear on D_2O exchange (Fig. 3). The shift of the singlet of C_1—OH in Fig. 3A is due to the concentration difference. A singlet at 4.7δ in Fig. 4B is due to HDO. The absence of a methyl group singlet between 1δ and 2δ and the presence of two triplets indicate that the indole nucleus is separated from C_1—OH by an ethylene linkage.

The nuclear magnetic resonance spectrum of β-ILA shows a multiplet signal similar to β-IEA centered at 7.4δ, corresponding to an indole nucleus (Fig. 5). The two quartet signals at 4.2δ (J 5Hz) and 3.2δ (J 5Hz) indicate that protons of $-\overset{H}{\underset{|}{C}}-$ and $-\overset{H}{\underset{H}{C}}-$ are coupled to each other. The signal at 2.5δ is due to Me_2SO.

The physicochemical and spectral properties of the isolated compounds are identical to authentic β-IEA and β-ILA (Sigma Chemical Co., St. Louis, Mo.). All *Candida* spp. tested produced both β-IEA and β-ILA (Table 2). *C. guilliermondii* and *C. krusei* produced more β-IEA and less β-ILA. The reverse is the case with the other three species. This pattern of producing either alcohol or hydroxy acid in excess is similar to the production of β-phenethylalcohol and its corresponding hydroxy acid, β-phenyllactic acid (7). The total yield of β-IEA and β-ILA ranged from 14 to 24% among *Candida* spp., as

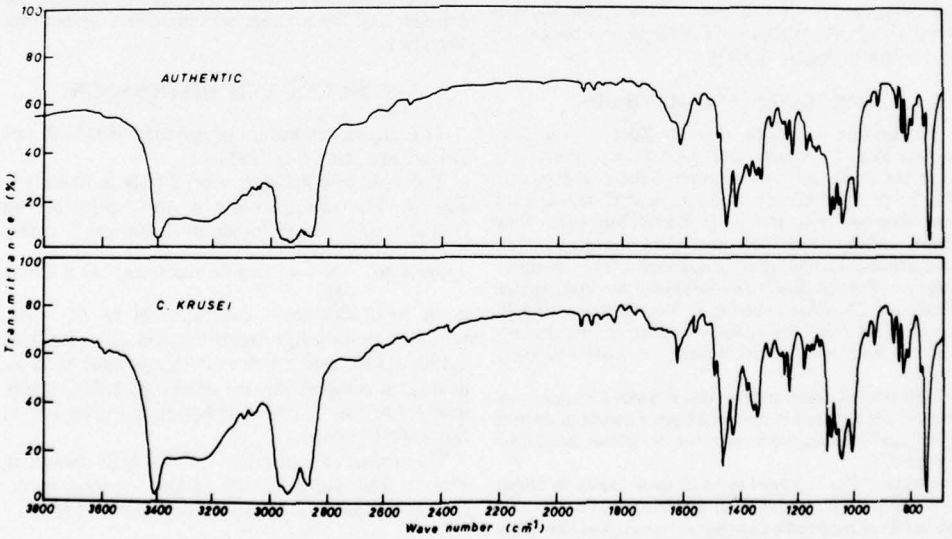

FIG. 1. *Infrared spectra of isolated and authentic β-IEA (tryptophol).*

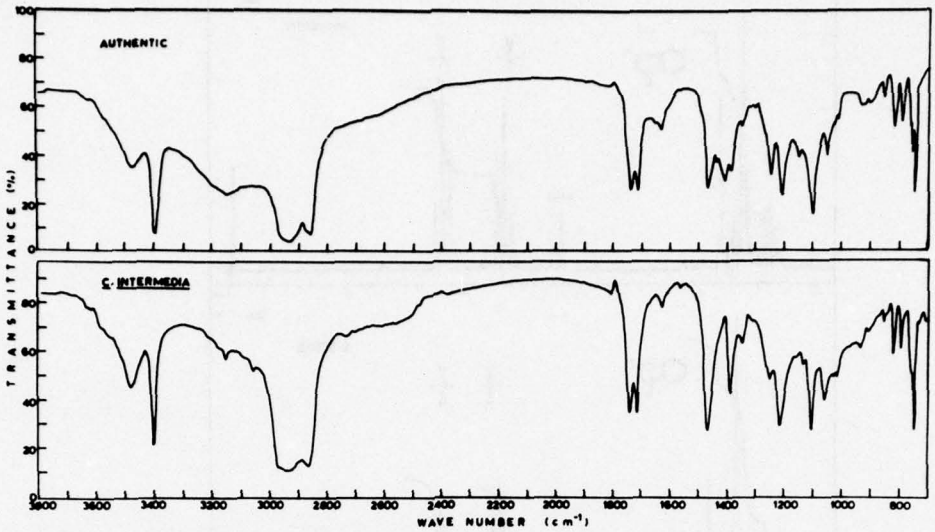

FIG. 2. *Infrared spectra of isolated and authentic β-ILA.*

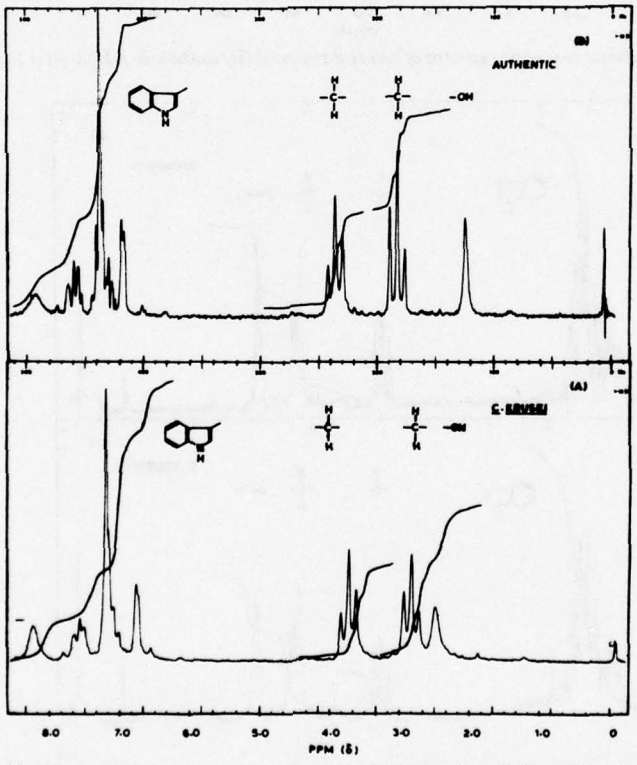

FIG. 3. *Nuclear magnetic resonance spectra of (A) isolated and (B) authentic β-IEA.*

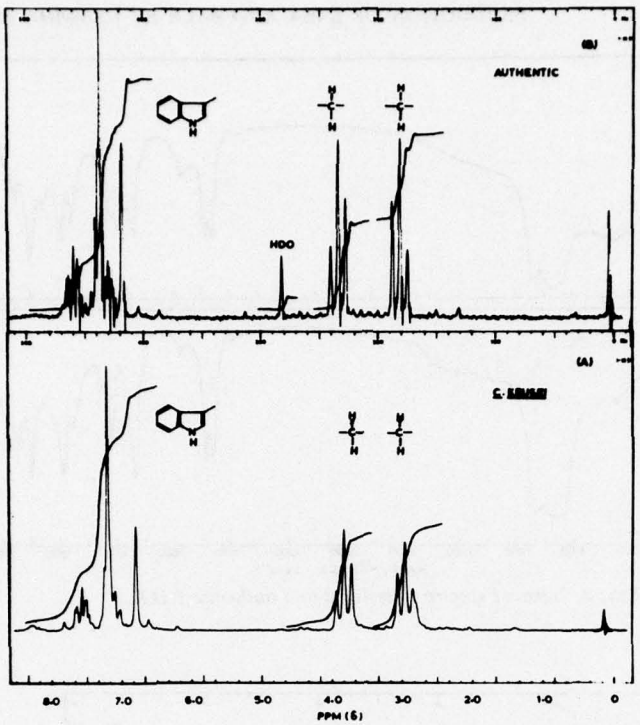

FIG. 4. *Nuclear magnetic resonance spectra of (A) isolated and (B) authentic β-IEA after D_2O exchange.*

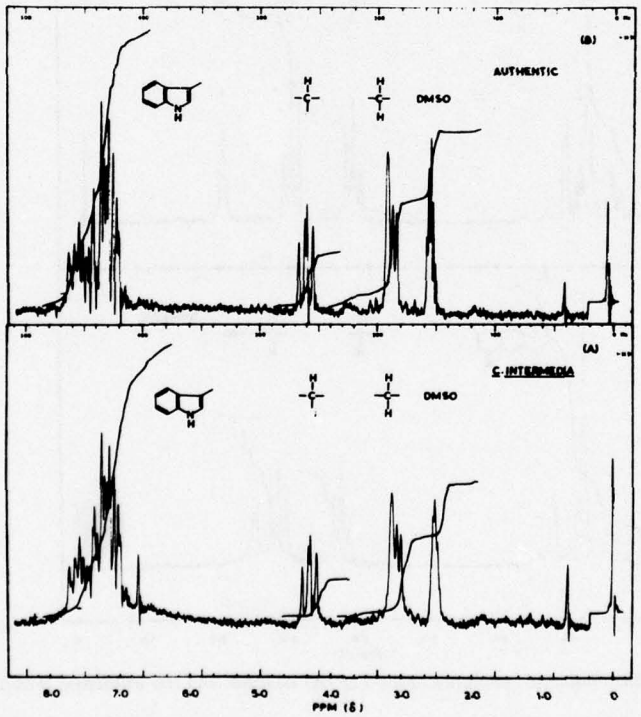

FIG. 5. *Nuclear magnetic resonance spectra of (A) isolated and (B) authentic β-ILA.*

compared to 34 to 67% of β-phenethylalcohol and β-phenyllactic acid together (7).

Labeled β-IEA and β-ILA have been isolated by using DL-[^{3}H]tryptophan (5T) (Table 3). Approximately 17% of the tryptophan taken up by the cells is found converted to β-IEA (specific activity, 0.21 μCi/mmol) and β-ILA (specific activity, 0.165 μCi/mmol). The specific activities of both labeled compounds remained the same after repeated purification.

β-IEA inhibited the growth of both gram-positive and -negative organisms in the concentration range of 6 to 12 mM (Table 4). However, gram-negative organisms in general are more susceptible than gram-positive organisms. Like β-IEA, β-ILA also inhibited the growth of *E. coli* at 6 mM and *B. cereus* at 15 mM.

The autoantibiotic property of β-IEA on *Candida* spp. is revealed by the data in Table 5. All species examined were totally inhibited at concentrations of 6 to 12 mM. However, *C. tropicalis*, which produces the lowest level of β-IEA, is highly susceptible to β-IEA as compared with other species. This was also observed with β-phenethylalcohol (6).

Some data illustrating the nature of inhibition exerted by β-IEA and β-ILA on the growth of *C. albicans* are shown in Fig. 6. When β-IEA was added to log-phase cells, no further growth took place. The inhibition of growth was seen as long as the cells were in contact with β-IEA. After washing the cells free of β-IEA, the cells exhibited a growth pattern similar to the control. This clearly shows the reversible nature of the inhibition by β-IEA. A similar effect is exhibited by β-ILA as well. The inhibition

TABLE 2. *Production of β-IEA and β-ILA by Candida species*

Yeasts	Yield (mg/liter of medium containing 1 g of L-trypotophan)[a]		
	β-IEA	β-ILA	% Conversion of L-tryptophan added
C. guilliermondii	93	62	15.5
C. krusei	132	50	18.2
C. intermedia	28	116	14.4
C. albicans	25	122	14.7
C. tropicalis	15	193	24.3

[a] The medium used consisted of: glucose, 20 g; KH_2PO_4, 3.5 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $CaCl_2 \cdot 2H_2O$, 2.5 g; L-tryptophan, 1 g; and biotin, 30 μg; distilled water to 1 liter. Cells were grown at 31 C for 2 days on a rotary shaker.

TABLE 3. *Isolation of [β-^{3}H]IEA and [β-^{3}H]ILA from culture filtrates of C. intermedia*[a]

Tryptophan	Counts/min
Amt added	3×10^6
Remaining after the extraction of culture filtrate	1.27×10^6
Taken up by the cells	1.73×10^6
Converted to:	
β-IEA (28.2 mg) 0.80×10^5	2.96×10^5
β-ILA (122.8 mg) 2.16×10^5	

[a] Cells were grown in the medium described in Table 2, which was supplemented with 10 μCi of DL-[^{3}H]tryptophan (5T) (1,600 mCi/mmol) obtained from BARC, Bombay, India. The compounds were isolated as described in the text, and radioactivity was measured in a Beckman LS-100 liquid scintillation counter. About 17% of the tryptophan taken up by the cells was found to be converted to β-IEA (4.6%) and β-ILA (12.4%).

TABLE 4. *Influence of β-IEA on the growth of gram-positive and gram-negative bacteria*

Organism	% Inhibition				
	3 mM[a]	6 mM	9 mM	12 mM	15 mM
Escherichia coli	12	60	90	100	100
Paracolobactrum aerogenoides	60	100	100	100	100
Proteus vulgaris	15	90	100	100	100
Aerobacter aerogenes	40	90	100	100	100
Bacillus subtilis[b]	25	50	75	100	100
Bacillus cereus[b]	25	25	50	75	100
Bacillus megaterium[b]	25	25	50	75	100

[a] β-IEA concentration.

[b] Since *Bacillus* spp. form pellicles, the growth inhibition is graded visually. Nutrient broth (5 ml) was inoculated with 0.1 ml of an 18-h culture and incubated at 37 C for 24 h. Growth was measured in a Klett-Summerson colorimeter at 540 nm.

TABLE 5. *Autoantibiotic effect of β-IEA on growth of Candida species*

Species	% Inhibition			
	3 mM[a]	6 mM	12 mM	18 mM
C. albicans	60	86	100	100
C. tropicalis	91	100	100	100
C. krusei	57	73	100	100
C. guilliermondii	68	88	100	100
C. intermedia	60	85	100	100

[a] β-IEA concentration.

[b] The medium used consisted of glucose, 20 g; KH_2PO_4, 3.5 g; $(NH_4)_2SO_4$, 2.5 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; $CaCl_2 \cdot 2H_2O$, 2.5 g; and biotin, 30 μg; in 1 liter of distilled water. A 5-ml amount of medium was inoculated with 0.1 ml of a 24-h culture and incubated at 30 C for 48 h. Growth was measured in a Klett-Summerson colorimeter at 540 nm.

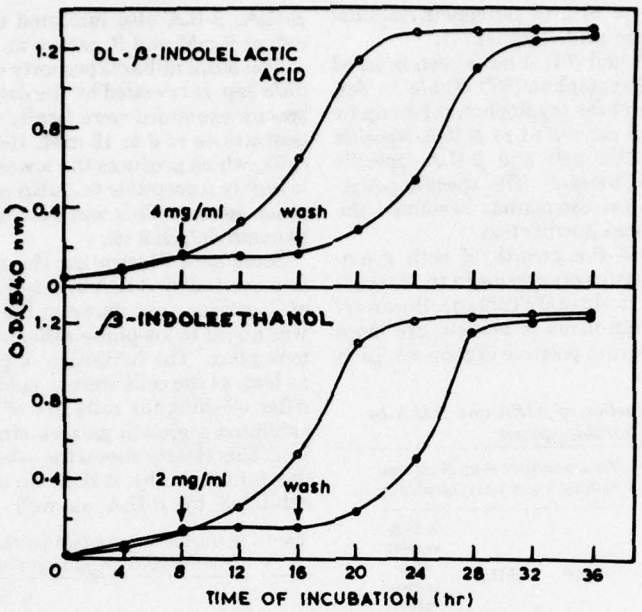

FIG. 6. *Effect of β-IEA and β-ILA on growth of C. albicans.*

caused by β-ILA may be due to its own effect or due to its conversion product. The conversion of β-ILA to β-IEA is revealed by isolating β-IEA from a culture filtrate of *C. albicans* incubated with β-ILA for 8 h. However, the antimicrobial effect of β-ILA by itself is evident from studies on gram-positive and -negative organisms. Thus, the autoantibiotic effect of β-ILA might be due to its conversion product but may also be considered as its own effect. The data presented reveal the ability of *Candida* spp. to convert a normal metabolite such as L-tryptophan to β-IEA and β-ILA. They are possibly biosynthesized via the following pathway:

L-Tryptophan →

indole pyruvic acid ⇄ indolelactic acid

↓

indole acetaldehyde → indoleethanol

The differences in the quantitative production of various alcohols from the corresponding amino acids may be due to the substrate specificity of the enzyme and/or to the autoinhibition exerted by the end products, namely, the alcohols concerned.

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BIOSYNTHESIS OF β-PHENETHYL ALCOHOL IN CANDIDA GUILLIERMONDII

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SUMMARY: Candida guilliermondii produced β-phenethyl alcohol and β-phenyllactic acid when grown in a synthetic medium containing L-phenylalanine as sole source of nitrogen. The cell-free preparations from these cells showed the following enzymes: phenylalanine aminotransferase, phenylpyruvate decarboxylase, phenylpyruvate reductase and phenylacetaldehyde reductase. The cell-free preparations of C. guilliermondii grown in medium with ammonium sulfate, lacked these enzyme activities, indicating the inducible nature of these enzymes. The results indicate the role of β-phenylpyruvate as a key intermediate in the pathway of biosynthesis of β-phenethyl alcohol and β-phenyllactic acid from L-phenylalanine.

INTRODUCTION

L-Phenylalanine is converted to either L-tyrosine or β-phenylpyruvate in bacteria (1,2), to cinnamate in most of the plants and fungi, and to tyrosine in mammalian systems. Thus, the pathway involved in the catabolism of L-phenylalanine varies widely in diverse organisms. However, in phenylketonuric patients, phenylalanine metabolism leads to accumulation of β-phenylpyruvate, β-phenyllactate and phenylacetylglutamine (3).

The biosynthetic abilities of different species of Saccharomyces and Candida have been exploited in the production of alcohols (4-7), vitamins (8), and enzymes (9). Candida albicans excretes β-phenethyl alcohol (β-PEA) when grown in Sabouraud's broth (10) or defined media (11). Several species of Candida have been reported to produce aromatic alcohols like β-PEA (11), β-(4-hydroxyphenyl)-ethanol (HOPEA) (12) and β-indoleethanol (β-IEA) (13) when L-phenylalanine, L-tyrosine or L-tryptophan

served as sole sources of nitrogen, respectively. Though the pathways involved in the biosynthesis of these aromatic amino acids in yeasts have been extensively studied, little is known about the enzymology of their catabolism. The enzymology of the biosynthesis and regulatory aspects of β-PEA and β-IEA are of great interest in view of their reported autoantibiotic activity (14). In this communication, we report the demonstration of the activities of the enzymes which convert L-phenylalanine to β-PEA and β-PLA in C. guilliermondii.

MATERIALS AND METHODS

Chemicals: L-Phenylalanine, β-phenylpyruvate, β-phenyllactate, β-phenethyl alcohol, α-ketoglutarate, NADH, NADPH, PLP, and TPP were obtained from Sigma Chemical Company, St. Louis, Mo, U.S.A. Phenylacetaldehyde obtained locally was purified by distillation.

Organism: Candida guilliermondii Z55 was obtained from the London School of Hygiene and Tropical Medicine, London. Stock cultures were maintained by bimonthly subculturing on Sabouraud's glucose agar slants.

Growth: The medium used for growing cells was of the composition: glucose, 2%; $(NH_4)_2SO_4$, 0.25% or phenylalanine, 0.1%; $CaCl_2.2H_2O$, 0.25%; $MgSO_4.7H_2O$, 0.25%; KH_2PO_4, 0.35%; and biotin, 3 μg/100 ml.

Erlenmeyer flasks of 500 ml capacity each containing 200 ml of medium were inoculated with 20 ml of 10 h culture of C. guilliermondii in the same medium, and grown for 12 to 24 h on a rotary shaker at 30±1°C. The cells were harvested by centrifugation in Sorvall RC2-B centrifuge at 4,000xg at 0-4°C. The cell-pellet was washed thrice with ice-cold 0.05M phosphate buffer, pH 7.0.

Cell-free preparations: Cells of C. guilliermondii were ground for 15-20 min with twice the amount of acid-washed sand (60-80 mesh) in a precooled mortar. The slurry was extracted with 0.05M Tris-HCl buffer, pH 7.4. The suspension was centrifuged at 18,000xg for 30 min. The supernatant was used to assay various enzyme activities. Protein content was estimated by the method of Lowry et al. (15).

Enzyme assays: Phenylalanine aminotransferase activity was assayed according to the procedure of Lin et al. (16).

Phenylpyruvate formed was estimated as its enol-borate complex, which absorbs at 300 nm.

Phenylpyruvate decarboxylase activity was assayed using standard manometric technique (17), by measuring carbon dioxide released.

Table 1. Enzymes involved in biosynthesis of β-phenethyl alcohol in Candida guilliermondii

Enzyme	Substrate	System	Specific activity
1. Phenylalanine aminotransferase[a]	L-Phenylalanine	Complete	210
		- α-Ketoglutarate	-
		- Phe	-
		- PLP	-
2. Phenylpyruvate decarboxylase[b]	β-Phenylpyruvate	Complete	47
		- TPP	-
		- Mg^{2+}	-
		- Substrate	-
3. Phenylpyruvate reductase[c]	β-Phenylpyruvate	Complete	18
4. Phenylacetaldehyde reductase[d]	Phenylacetaldehyde	Complete	1600

a. Complete system, in a final volume of 2.5 ml, contained L-phenylalanine, 30 μmoles; α-ketoglutarate, 30 μmoles; pyridoxal phosphate, 10 nmoles; potassium phosphate buffer, pH 8.0, 250 μmoles; and enzyme protein (1.5 mg). Assay temperature was 37°C.

b. Complete system, in a final volume of 3.2 ml, contained β-phenylpyruvate, 20 μmoles; $MgCl_2$, 1 μmole; thiamine pyrophosphate, 2 μmoles, potassium phosphate buffer, pH 6.0, 1 mmole; and enzyme protein (1.5 mg). Assay temperature was 37°C.

c and d. Complete system, in a final volume of 2 ml, contained β-phenylpyruvate, 20 μmoles, or phenylacetaldehyde, 10 μmoles; NADH or NADPH, 0.2 μmoles; potassium phosphate buffer, pH 6.5, 50 μmoles; and enzyme protein (0.5 mg). Assay temperature was 25°C.

The activities of phenylpyruvate reductase and phenylacetaldehyde reductase were assayed by determining the decrease in absorbancy of reduced pyridine nucleotides at 340 nm in a Carl Zeiss spectrophotometer.

One unit of activity is defined as equivalent to that amount of the enzyme which catalyzes the conversion of one nmole of substrate per minute. Specific activity is expressed as units of enzyme activity per milligram protein.

RESULTS AND DISCUSSION

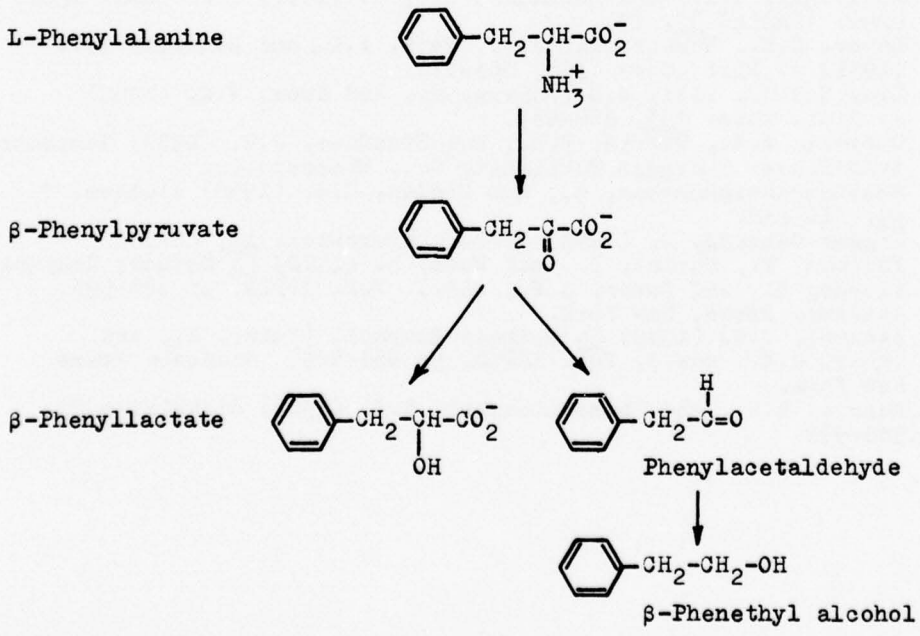
The ability of C. guilliermondii to grow in synthetic media containing L-phenylalanine as sole source of nitrogen and to produce β-PEA has been reported earlier from our Laboratory (11). Cell-free preparations from these cells showed phenylalanine aminotransferase (PAT) activity (Table 1). The enzyme displayed an absolute requirement for pyridoxal phosphate for its activity. The occurrence of PAT in several organisms (1) including yeasts viz., S. cerevisiae (18), and in S. fragilis (19) was reported and its role in the metabolism of L-phenylalanine reviewed (1). Phenylpyruvate decarboxylase which catalyzes the non-oxidative decarboxylation of β-phenylpyruvate to phenylacetaldehyde, was also detected in the cell-free preparations. It required both thiamine pyrophosphate and Mg^{2+} for its activity. The occurrence of this enzyme in bacteria was reported (20). Cell-free preparations of C. guilliermondii also showed activity of phenylpyruvate reductase which catalyzes the reduction of β-phenylpyruvate to β-PLA, and the reaction is reversible. Both NADH and NADPH served as electron donors. The product of the reaction catalyzed by this enzyme. viz., β-PLA was isolated and identified by paper chromatography (11). The presence of this enzyme was also reported in phenylketonurics, who excrete β-PLA, L-phenylalanine, β-phenylpyruvate and phenylacetylglutamine in urine (21). Conversion of β-phenylpyruvate to β-PLA has not so far been reported in microorganisms including yeasts.

Phenylacetaldehyde reductase which reduces phenylacetaldehyde to β-PEA requiring either NADH or NADPH for its activity was also detected in cell-free preparations of C. guilliermondii. Though a possible mechanism of its conversion in plants has been

proposed by earlier workers (22), its activity has not been demonstrated. However, an enzyme converting p-hydroxyphenylacetaldehyde to tyrosol in S. cerevisiae was reported (18).

Cell-free preparations of C. guilliermondii grown on ammonium sulfate as nitrogen source failed to show these enzyme activities indicating the inducible nature of these enzymes. The results presented here indicate the role of β-phenylpyruvate as a key intermediate in the conversion of L-phenylalanine to β-PEA and β-PLA by the pathway proposed below. A similar pathway might be operating in the biosynthesis of HOPEA and β-IEA from L-tyrosine and L-tryptophan, respectively, in Candida species. Studies on purification and characterization of these enzymes, and their role in the regulation of the pathway are in progress.

The proposed pathway for the biosynthesis of β-phenethyl alcohol and β-phenyllactate from L-phenylalanine in Candida guilliermondii is as follows:



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6c. Regulation of phenylalanine aminotransferase activity in Candida guilliermondii

Phenylalanine aminotransferase, the first enzyme of the pathway in the biosynthesis of PEA, converts L-phenylalanine to β-phenylpyruvic acid and is induced by L-phenylalanine by 15-20 fold. The enzyme was maximally induced by 6 hr with an optimum concentration of the inducer at 6 mM. The fact that the induction is blocked completely by cycloheximide at as low a concentration as 10^{-4}M, indicates that the enzyme is synthesized de novo. Removal of glucose from the induction medium reduced the activity by 60%. Induced synthesis of the enzyme is inhibited completely by 10^{-3}M each of azide, DNP, cyanide and antimycin A. These results reveal that the induction of the enzyme is energy-dependent. Metabolites of L-phenylalanine viz., phenylpyruvic acid, phenylacetaldehyde, PEA and PLA have been tested at 1 mM, 8 mM and 10 mM concentrations for their repression on the enzyme synthesis. Among these only PLA at 10^{-3}M concentration showed over 90% repression of enzyme synthesis. Present results explain the regulatory properties of phenylalanine aminotransferase activity and how its activity is controlled by the concentration of PLA.

6d. Regulation and properties of nitrate reductase in Candida utilis

A study was undertaken to delineate the enzymological basis of nitrate assimilation in yeasts and to gain further insight into and understanding of this

complex process as a whole. Emphasis was laid on the regulatory aspects of nitrate reductase in order to explain the subtle differences it may possess from the enzyme from other sources.

Candida utilis known as 'Food/Fodder yeast', was chosen for the study as it provides an ideal system for investigation on nitrate assimilation by virtue of its structural simplicity, non-pathogenicity, rapid growth, adaptability to a wide range of conditions and above all, for its ability to readily utilize a variety of nitrogen sources, including nitrate (La RMe, T.A., and Spencer, J.F.T., 1968, Can. J. Microbiol. 14, 79-86).

Aspects investigated include: Decryptification of nitrate reductase activity (pages, 108-111); its characterization (pages, 112-122); its regulatory properties (pages, 123-130) and its molecular basis of induction (pages, 131-135).

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DECRYPTIFICATION OF NITRATE REDUCTASE ACTIVITY IN THE YEAST *CANDIDA UTILIS*

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ABSTRACT

A diverse variety of chemicals and drugs are known to affect the cell walls of bacteria and yeasts resulting in an enhanced access of the intracellular enzymes to the added substrates. The ability of organic solvents, antifungal drugs, detergents, and chelating compounds to decryptify NAD(P)H : nitrate oxidoreductase (EC 1.6.6.2) activity in *Candida utilis*, has been investigated. Of these, toluene, butanol, 2-phenylethanol and amphotericin B, in that order, are effective. The degree of decryptification is dependent on the concentration of the agent, and at 2·5% (v/v), toluene-ethanol elicited highest enzyme activity. These permeated cell preparations greatly aid in the regulatory studies of various yeast enzymes *in situ*.

INTRODUCTION

INVESTIGATIONS concerning regulatory phenomena, *viz.*, induction and repression of various enzymes such as β-galactosidase in *Escherichia coli* were carried out *in situ* using permeated bacterial cells[1-3], thus doing away with the laborious process of preparing cell-free extracts. The growing awareness in the recent past of the fallacies inherent in inferring the *in vivo* situation from *in vitro* observations further strengthened the surge of interest in the development of novel methods to decryptify various enzymes and assay the activities *in situ* in permeated cells of a variety of organisms[4-10].

The detailed studies on the regulation of yeast nitrate reductase (EC 1.6.6.2) (NAR) under investigation in our laboratory which involve determination of rapidly changing enzyme levels[8], necessitated the development of an alternate assay method, which is simpler, rapid and more sensitive than the conventional *in vitro* procedure.

The paper is concerned with the examination of the relative efficacy of different compounds in decryptifying NAR activity in induced *Candida utilis* cells. The typical method used to assay the decryptified NAR activity *in situ* using permeated *C. utilis* cells is also described.

MATERIALS AND METHODS

Chemicals.—Lab reagent grade benzene was purchased from Sarabhai M. Chemicals, and solvent ether from Alembic Chemical Works Co. Ltd., Baroda. Analytical reagent grade chloroform, DMSO, DMF, acetone, propanol, butanol, amyl alcohol, potassium nitrate, sulfur-free grade lab reagent toluene and polyethylene glycol 400 were purchased from BDH Ltd., Bombay, India. 2-Phenylethanol, amphotericin B, nystatin, griseofulvin, sodium salt of EDTA, NADH, FAD, DTT, and crystalline bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, U.S.A. Miconazole (1-{2-(2, 4-dichlorophenyl)-2-[(2, 4-dichlorophenyl) methoxy]ethyl}-1 H-imidazole mononitrate) was a kind gift from Janssen Pharmaceutica, B-2340, Beerse, Belgium, through the courtesy of Ethnor, Ltd., Bombay. Sulfanilamide was purchased from the Indian Drugs and Pharmaceuticals, Ltd., India and N-(1-naphthyl) ethylenediamine. 2 HCl from BDH, Ltd., Poole, England. Other chemicals were of analytical grade from BDH Ltd., Bombay, India or Riedel-De Haenag Seelze-Hannover, Germany.

Organism and Maintenance.—*Candida utilis* CBS 4511 was obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. The yeast was maintained by subculturing once in two months on Sabouraud's glucose agar slants containing neopeptone, 1% ; glucose, 1% and yeast extract, 0·2%.

Media and Cultivation Techniques.—The basal medium was essentially the same as described by Wickerham[11] with a few modifications, and contained potassium nitrate, 50 mM (N) as nitrogen source (induction medium). The yeast was cultured by inoculating liquid medium in 500 ml-Erlenmeyer flasks with 5% (v/v) of 24 h culture, aerated by shaking on an Emenvee gyratory shaker (180 rpm) at 30° C. The cells in late exponential phase of growth ($\simeq$ 12 h after inoculation) were harvested, washed with chilled phosphate buffer, and packed by centrifugation at 6,000 × g for 5 min. at 4° C in a Sorvall RC 2-B centrifuge. The packed cells were stored frozen at − 10° C till use.

Induction of NAR.—The cells of *C. utilis* grown on KNO_3 (1%, w/v) as nitrogen source had high levels of NAR activity. The inducibility of NAR in *C. utilis* by NO_3^- was reported[12].

Preparation of Permeated Yeast Cells.—The general procedure of permeating the yeast cells was as follows : An aliquot of 0·05 ml chilled

decryptifying agent was added to 2 ml of the cell suspension [100 mg (fresh wt) of induced cells/ml of 'preparation buffer', 0·1 M phosphate buffer, pH 7·0 containing 10^{-4} M DTT and $1·5 \times 10^{-4}$ M EDTA] in a 16 mm test-tube, and shaken for 2 min at 4° C on a Jay Vortex Mixer. Suitable aliquots of this preparation were used to assay NAR *in situ*.

Enzyme Assay :

NAR in situ.—The reaction mixture contained, KNO_3, 0·02 M ; 0·5 ml (50 mg fresh wt) of permeated cell preparation, and 0·1 M pyrophosphate buffer, pH 7·0 to give a final volume of 1 ml. The reaction was carried out 10 min. at 37° C and terminated by the addition of 1 ml of 1% sulfanilamide in 3 N HCl. The nitrite content of an aliquot of the supernatant obtained after low speed centrifugation was determined. There was no significant reduction of nitrite in blanks under the assay conditions.

Analytical Assay :

Nitrite.—The nitrite content of 1 ml-reaction mixture was determined colorimetrically by a modification of the diazo coupling procedure described by Snell and Snell[13], with 1 ml of 1% (w/v) sulfanilamide in 3 N HCl followed by 1 ml of 0·02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride, and distilled water to give a final volume of 5 ml. After 10 min, the color intensity was determined at 540 nm with a Beckman model DU spectrophotometer. The absorbancy values were converted into nmoles of nitrite by multiplication with the slope of a nitrite concentration reference curve made with sodium nitrite.

Units and Specific Activity.—One unit of activity is defined as equivalent to that amount of the enzyme which catalyzes the formation of 1 nmole of nitrite per 1 min and the specific activity is expressed as units per mg of total extractable protein (55 mg/g fresh yeast).

Results

Relative Efficacy of Various Permeating Agents in Decryptifying NAR Activity in C. utilis.—To select a suitable permeating agent that can effectively decryptify NAR in *C. utilis,* cells from exponential phase of growth were harvested, washed, and suspended in the preparation buffer to give 100 mg (wet wt) of cells/ml, and treated with various permeating agents reported in the literature, such as different organic solvents including alcohols, membrane-affecting drugs, chelating agents, etc., according to the typical procedure, described under Materials and Methods. A comparison of the relative levels of NAR activity of the cells permeated by different solvents showed toluene to be the most effective, and benzene the next best (Table I). Acetone, DMSO, caloroform, ether and DMF were not efficient as permeating agents.

Table I

Decryptification of nitrate reducatase activity in C. utilis *by various compounds*

Solvent[a]	NAR[c] specific activity
Organic solvents:	
Toluene	18 0
Benzene	9 9
Acetone	1·1
DMSO	0·1
Alcohols:	
Butan-1-01	16·2
2-phenylethanol	14·7
Ethanol	13·2
Polyethylene glycol 400	7·8
Propan-2-ol	5·4
Membrane-active drugs[b]:	
Amphotericin B	12·0
Miconazole	8 0

a used at a concentration of 5% (V/V).
b The final concentration was 1 mM
c Assayed *in situ* at 37 °C for 10 min.

Butanol, followed by ethanol, was the best among the aliphatic alcohols tested in decryptifying NAR activity. Phenylethanol, the only aromatic alcohol tested was almost as effective as butanol. Although polyethylene glycol 400 and propanol did show some effect, the relative levels of NAR activity decryptified, however, were not significant.

A number of antifungal drugs, known to affect cell membranes of the susceptible organisms, were also tested for their efficacy in permeating yeast cells. Amphotericin B was most effective folowed by miconazole, a synthetic compound recently coming into increasingly wide use as a broad-spectrum antimicrobial drug. Nystatin and griseofulvin, at the concentrations tested, had little effect. Various detergents like Triton X-100, Brij, sodium deoxycholate either failed to bring about any decryptification of NAR activity or inactivated the enzyme. Similarly, EDTA[5] and bovine serum albumin[7] used by earlier workers as permeating agents, were not useful in decryptifying NAR activity of *C. utilis* cells.

Effect of Concentration of the Permeating Agent on Decryptification of NAR Activity.—Further, the effect of varying the concentration and composition of the permeating agent on decryptification of NAR activity was investigated. Figure 1 shows the effect of different concentrations of toluene, ethanol and their mixture on permeation of the yeast cells. Although toluene was very effective in permeating the yeast cells, its effect was enhanced significantly, when it was used in combination with ethanol, as indicated by increased levels of NAR activity. Maximal enzyme activity was elicited by 2·5% (v/v) toluene-ethanol (1 : 4, v/v). Higher concentrations of toluene-ethanol, however, were deleterious to the enzyme activity[14]. This could be due to the probable sensitivity of the enzyme to alcohol because toluene, by itself did not affect NAR activity at higher concentrations. Untreated cells, however, showed no evidence of decryptified NAR activity.

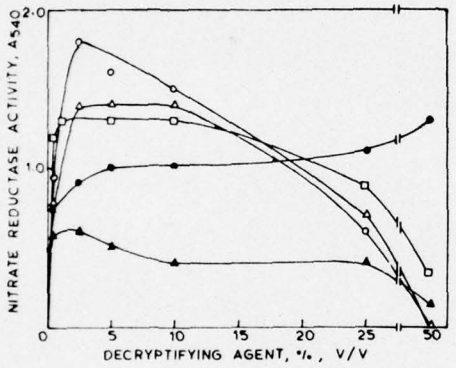

FIG. 1. Decryptification of nitrate reductase activity in *C. utilis* as a function of the concentration of the permeating agent. An O.D. of 1·0 at 540 nm corresponds to 10 nmoles of nitrite formed/min/mg protein. (●) toluene; (▲) ethanol; (□) toluene-ethanol, 1 : 1, v/v; (△) toluene-ethanol, 1 : 2, v/v; (○) toluene-ethanol, 1 : 4, v/v.

DISCUSSION

Of various permeating agents examined, toluene, in combination with alcohol, was most effective in decryptifying NAR activity in *C. utilis* cells. A variety of substances including toluene[1–3,16,17] benzene[6], DMSO[8], polyene antibiotics[4], basic proteins[7], and chelating compounds[5], was reported to permeate bacterial and yeast cells. Toluene found extensive use in such studies for its selective action on the cells rendering them freely permeable to a wide variety of low molecular weight substances[16,17]. Our results substantiate this observation regarding the high retention of the enzymatic potential of the treated cells. The absence of NAR activity in cells treated with EDTA (widely used by other workers to permeate cells) may be the result of the inhibition by EDTA of the activity of NAR, a metalloflavo-protein rather than its failure to permeate yeast cells[14].

The toluene-treated cells showed high NAR activity under *in situ* conditions without added cofactors[19], contrary to the property of NAR *in vitro*, where the enzyme showed an absolute requirement for NAD (P) H[14,15]. However, the addition of coenzymes increased the *in situ* activity by 50%. The initial high *in situ* activity could be due to the participatiion of endogenous NAD (P) H and FAD in the reaction. Presence of adequate intracellular concentrations of NADH and cAMP in yeast cells was recently reported by Gancedo and Gancedo[18].

The proposed *in situ* assay, thus, is much simpler, less expensive, quite sensitive and reliable, and enables one to overcome the apparent shortcomings attributed to the *in vitro* method of assay. The additional advantage that the cells need no other supplement of the coenzymes for routine assay of the enzyme activity *in situ*, makes this procedure uniquely distinct even over the other *in situ* assay methods known, where the only advantage over the conventional *in vitro* methods is that whole cells can be used in place of cell-free preparations. All our subsequent studies on the regulatory phenomena controlling the biosynthesis of NAR in *C. utilis*, which involve handling of innumerable concentrations, and assay of NAR activities at brief time periods, are conducted using the *in situ* technique and the results appear elsewhere.

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CHARACTERIZATION OF NITRATE REDUCTASE FROM THE YEAST *CANDIDA UTILIS*

By

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Characterization of nitrate reductase from the yeast *Candida utilis*

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Abstract

The assimilatory nitrate reductase [NAD (P) H: nitrate oxidoreductase, EC 1.6.6.2] of the nitrate-utilizing yeast *Candida utilis* was prepared in soluble form from cells grown aerobically with nitrate as the nitrogen source, and some of its properties studied. The enzyme is cytoplasmic in intracellular distribution and its activity is NAD (P) H-dependent. The enzyme utilized both NADH and NADPH as electron donors but not reduced viologen dyes, flavins and other reductants. Flavin compounds, at higher concentrations, decreased the enzyme activity, while at very low concentrations, FAD (10^{-5} M) showed little stimulation.

The optimal activity of the enzyme was obtained when the reaction was carried out for 10 min at 37° C at pH 7·0 with 20 mM KNO_3, 0·1 mM NAD (P) H, 0.01 mM FAD and 140 μg enzyme protein.

Complete loss of enzyme activity was observed on exposure to 50° C for 3 min. pHMB, cyanide, azide and EDTA, in that order, inhibited the enzyme activity effectively. The inhibition caused by pHMB was largely reversed by DTT.

1. Introduction

Nitrate reductase, which catalyzes the first step in the assimilatory reduction of nitrate, *viz.*, conversion of nitrate to nitrite, attracted best attention for its biosynthetic complexity[1–6] and mechanistic intricacies.[7–9] It has been well characterized in plants, algae, fungi and bacteria.[9–11] On the other hand, the information on this enzyme from nitrate-utilizing yeasts is yet scarce,[12,13] except for the preliminary investigations by Silver,[14] and

Pichinoty and Metenier[15,16] on *Hansenula anomala*. These studies indicated that nitrate reductase (NAR) from *H. anomala* is an inducible metalloflavoprotein, similar in all respects to that of *Neurospora crassa* except for the difference in the specificity for reduced pyridine nucleotides. Later, Rivas *et al.*,[12] working with *Torulopsis nitratophila*, corroborated this observation. Further, these authors reported that the enzyme from *T. nitratophila* exists in active and inactive forms, which are interconvertible, and to efficiently use NADH, MVH and BVH as electron donors for its activity.

Recently, interest in this enzyme from yeasts has been revived with intensive investigations initiated in our laboratory on assimilation of inorganic nitrogen compounds by various yeasts including the food yeast *Candida utilis*. The occurrence of inducible nitrate reductase in the cells of *C. utilis* grown in presence of nitrate was reported.[17] Detailed studies were conducted on various aspects of the regulatory phenomena that control the induction of nitrate reductase in *C. utilis*.[18]

We report in the present paper the preparation and *in vitro* characterization of a soluble NAD (P) H-nitrate reductase from *C. utilis* grown on nitrate and compare its properties with those from other systems, already reported in the literature.

2. MATERIALS AND METHODS

CHEMICALS

NADH, NADPH, FMN, FAD, glutathione (reduced form), pHMB, β-mercaptoethanol, L-cysteine, DTT and methyl viologen were obtained from Sigma Chemical Company, U.S.A.; sulfanilamide from Indian Drugs and Pharmaceuticals Ltd., Hyderabad; N-(1-naphthyl) ethylenediamine dihydrochloride and benzyl viologen from BDH Limited, England, and neopeptone and yeast extract from Difco Laboratories, Detroit, U.S.A. KNO_3, hydrazineH_2SO_4, ammonium sulfate and KH_2PO_4 were analytical reagents, and $NH_2OH.HCl$, urea and EDTA lab reagents from BDH Ltd., Bombay. Analytical grade $NaNO_2$ was purchased from Sarabhai M. Chemicals Ltd., Baroda, K_2HPO_4 from J. T. Baker Chemical Co., New Jersey, U.S.A., and sodium dithionite from E. Merck A. G. Darmstadt, Germany. Sodium pyrophosphate was obtained from Reidel-de Haenag, Seelze-Hannover, Germany.

BUFFERS

Buffer solutions were prepared by the method of Gomori[19] using potassium phosphate salts.

"*Preparation buffer*": 0·1 M Phosphate buffer, pH 7·0, with 0·15 mM EDTA and 0·1 mM DTT.

"*Washing buffer*"; 0·001 M Phosphate buffer, pH 7·3 with 0·15 mM EDTA and 0·1 mM DTT.

Medium

The basal liquid medium for routine cultivation of yeasts was prepared essentially according to the formula of Wickerham.[20] KNO_3 was used as nitrogen source and manganese, 400 μg/L and molybdenum 500 μg/L were additional inclusions.

Organism and maintenance

Candida utilis CBS 4511 was obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. The yeast was subcultured every two months and maintained by transfer and growth on Sabouraud's glucose agar slants containing 1% neopeptone, 1% glucose and 0·2% yeast extract.

Cultivation of the yeast

The yeast cells from a slant grown for 24 h were suspended in 2 ml of sterile distilled water and 75×10^9 cells used to inoculate 200 ml of the medium contained in 500 ml Erlenmeyer flasks. The flasks were then shaken for 12 hrs on an Emenvee gyratory shaker (180 rpm) at 30° C.

Cell-free preparations

The yeast cells thus grown in presence of nitrate were harvested, washed with ice-cold distilled water and washing buffer, and packed by centrifugation at 4° C. The cells were then homogenized by grinding for 15 min at 4° C with three times their weight of chilled carborundum and extracted with three volumes of cold preparation buffer. The supernatant, obtained by centrifugation of the slurry for 20 min at 20,000 $\times$g in a refrigerated RC2–B Sorvall centrifuge, was used to assay the activities of various enzymes *in vitro*.

Enzyme assays

NAD(P)H-nitrate reductase (NAR): NAD(P)H-nitrate oxidoreductase (EC 1.6.6.2) activity was assayed essentially according to the procedure described by Nason and Evans.[21] The assay mixture in a final volume of 1 ml contained pyrophosphate buffer, pH 7·0, 0·04 M; KNO_3, 0·02 M; FAD, 0·01 mM; NAD (P) H, 0·10 mM; and sufficient enzyme

to result in the formation of 5–40 nmoles of nitrite. The reaction was carried out for 10 min at 37° C, terminated by the addition of 1 ml of 1% (w/v) sulfanilamide in 3N HCl, and the nitrite formed was estimated. A zero-time control was included to correct for turbidity caused by the enzyme. One unit of activity is defined as equivalent to that amount of the enzyme which catalyzes the formation of 1 nmole of nitrite per minute, and the specific activity is expressed as units per milligram protein.

REDUCED FLAVIN NUCLEOTIDE-NITRATE REDUCTASES (FNH_2-NAR): The reaction mixture for determination of FNH_2-NAR activities consisted of KNO_3, 0·02 M; FAD/FMN, 0·01 mM; enzyme, 0·1 ml ($\simeq$ 1 mg protein); 0·03 ml of a freshly prepared solution of dithionite (8 mg per ml of 0·8% $NaHCO_3$) and 0·1 M phosphate buffer, pH 7·0 to give a final volume of 0·5 ml. The reaction was started by the addition of dithionite and was allowed to proceed for 10 min. The assay mixture was essentially anaerobic under these conditions, owing to the presence of dithionite. The reaction was stopped by vigorous shaking of the test tube on a Jay Vortex Mixer to destroy dithionite and the nitrite formed was determined. The results obtained were corrected for values obtained in identical reaction mixtures shaken at zero time. The activity units are the same as in the case of NAD (P) H-NAR.

REDUCED VIOLOGEN-NITRATE REDUCTASES (MVH-NAR/BVH-NAR): Essentially the same procedure of assay was followed for determining MVH–NAR/BVH–NAR activities as for FNH_2–NAR. Methyl viologen/benzyl viologen (10^{-4} M) replaced FAD, and 0·02 ml, instead of 0·03 ml, of dithionite solution was added to initiate the reaction. The activity units are the same as in the case of NAD (P)H-NAR.

ANALYTICAL ASSAYS

PROTEIN: Protein was estimated by the Biuret method,[22] with crystalline bovine serum albumin as standard.

NITRITE: The nitrite content of 1 ml reaction mixtures was determined colorimetrically by a modification of the diazo coupling procedure described by Snell and Snell,[23] with 1 ml of 1% (w/v) sulfanilamide in 3N HCl, followed by 1 ml of 0·02% (w/v) N-(1-naphthyl) ethylenediamine and distilled water to give a final volume of 5 ml. After 10 min, the color intensity was determined at 540 nm with a Beckman model DU spectrophotometer. The absorbancy values were converted into nmoles of nitrite by multiplication with the slope of a nitrite concentration reference curve made with sodium nitrite.

The presence of added reduced pyridine nucleotides at usual enzymatic assay levels did not interfere with color development to warrant their removal. However, in the cases where high levels of NADH and NADPH were used, like during determination of substrate saturation, the residual excess amounts of the coenzymes were precipitated by adding 0·2 ml of 1 M zinc acetate, and removed prior to the measurement of nitrite.

3. Results

Intracellular localization

Cells of *C. utilis* grown in presence of KNO_3 were ground with carborundum, fractionated by differential centrifugation and NAR activity of various fractions was determined (table 1). Bulk of the total NAR activity was found in cytosol (S–100), indicating the soluble nature of this enzyme.

Substrate specificity

Requirement and specificity for exogenous electron donor: Nitrate reductase showed an absolute requirement for a reduced pyridine nucleotide for its activity (table 2). NAR activity obtained with NADH as electron donor was 2·0 to 2·5-fold higher than that observed with NADPH. Activities of FNH_2-NAR, MVH-/BVH-NAR were not detectable. None of the other reductants tested could serve as electron donor for NAR activity (table 3).

Specificity and requirement of added flavin compounds: Omission of FAD from assay system did not result in decreased enzyme activity, irrespective of whether NADH or NADPH was the electron donor (table 2). The influence of various flavin compounds on NAR activity is shown in table 4. At 10^{-4} M concentration, riboflavin, FMN and FAD, in that order lowered the enzyme activity significantly. Hence, the effect of FAD was examined over a wide range of concentrations. Added FAD, however, failed to stimulate NAR activity.

Various other enzymatic properties of NAR, optimal pH, temperature, substrate concentration, etc., as determined with S–20 preparation of the enzyme filtered through Sephadex G–25 column, previously equilibrated with preparation buffer are presented in table 5.

Thermal stability

Figure 1 portrays the thermal stability of the catalytic activity of NAD (P) H-NAR from *C. utilis*. A rapid loss of activity occurred on exposure of the enzyme to 50° C for 3 min.

Table 1. Intracellular localization of nitrate reductase in *C. utilis*

Fraction	Volume (ml)	Total protein (mg)	NAR activity	
			Total units	Recovery (%)
A. Crude homogenate	68	918	10,280	100·0
B. 1,500 × g supernatant	66	478	10,319	100·4
C. 20,000 × g supernatant	63	463	11,898	115·7
D. 20,000 × g pellet	2	5	38	0·4
E. 104,000 × g supernatant	58	362	12,755	124·1
F. 104,000 × g pellet	4	16	18	0·2

Fresh cells of *C. utilis* (25 g) were homogenized and cell-free extracts prepared as described under Materials and Methods. The crude homogenate, diluted to 70 ml with precooled preparation buffer and designated fraction A, was centrifuged for 10 min at 1,500 × g, and the pellet (unbroken cells and cell debris) was discarded. The supernatant (Fraction B) was centrifuged for 30 min at 20,000 × g to give supernatant (fraction C) and a particulate (mitochondria, etc.) pellet (fraction D). Fraction C was further centrifuged for 1 hr at 104,000 × g to yield supernatant (fraction E) and a pellet (fraction F). The enzyme assays were carried out according to the typical method described under Materials and Methods.

The higher levels of NAR activity recovered in fractions C and E may be due to the removal of inhibitor(s) present in the crude homogenate (fraction A).

Table 2. Influence of various cofactors on the activity of *C. utilis* nitrate reductase

Assay system	NAR[a] specific activity
Complete (NADH + FAD) ..	15·6
FAD omitted ..	14·0
NADH omitted	*
Complete (NADPH + FAD) ..	7·6
FAD omitted ..	5·3
Minus cofactors ..	*

[a] Assayed *in vitro* at 37° C for 10 min as described in the text.

* Not detectable.

Table 3. Suitability of various reductants as electron donors in reduction of nitrate by *C. utillis* nitrate reductase

Reductant (1 mM)	NAR[a] specific activity
NADH ..	15·2
NADPH ..	7·6
Dithionite ..	*
Borohydrate ..	*
β-Mercaptoethanol ..	*
Dithiothreitol ..	*
Glutathione (reduced) ..	*
Cysteine ..	*
Ascorbic acid ..	*
Formate ..	*
Succinate ..	*
Malate ..	*
Lactate ..	*

[a]Assayed *in vitro* at 37° C for 10 min as described in the text; NAD (P) H was substituted with the reductant under investigation.
* Not detectable.

Table 4. Influence of added flavin compounds on nitrate reductase activity from *C. utilis*

Flavin compound added	Concentration (M)	NAR[a] specific activity
Nil (control) ..	..	14·0
Riboflavin ..	10^{-4}	8·1
FMN ..	10^{-4}	9·0
FAD ..	10^{-3}	6·1
	10^{-4}	10·7
	10^{-5}	15·6
	10^{-6}	14·7
	10^{-7}	14·0
	10^{-8}	10·2

[a] Assayed *in vitro* at 37° C for 10 min as described in the text. The electron donor was NADH 10^{-4} M, and the flavin concentration was varied as mentioned in the table.

Table 5. *Summary of optimal conditions for assay of C. utilis nitrate reductase activity in vitro*

Property		Optima
pH	..	7·0
Temperature	..	37° C
Substrate (NO_3^-) concentration	..	20 mM
Specificity for electron donor	..	NADH/NADPH
Concentration	..	0·1 mM
Electron carrier	..	FAD
Concentration	..	0·01 mM
Protein (enzyme) concentration	..	140 μg
Reaction time	..	10 min

The enzyme reaction was carried out *in vitro* at 37° C for 10 min as described in the tex except for the particular condition under investigation, which was varied over a wide range for determination of the optima. NADH was the electron donor in the assays in determining the above optima except for the electron donor.

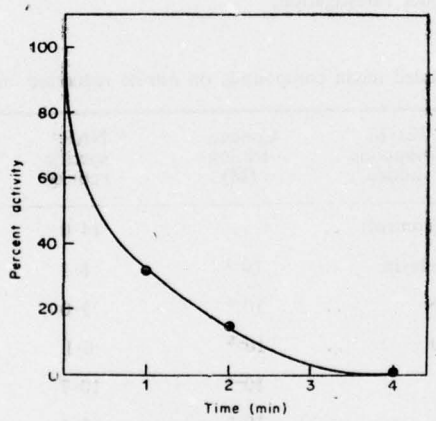

Figure 1. Effect of heating on nitrate reductase activity from *C. utilis*. A sample of 2·0 m of the cell-free preparation passed through Sephadex G–25 column previously equilibrated with reparation buffer contained in a test tube, 13 × 100 mm, was placed in a water-bath at 50° C pAt the indicated intervals of time, 0·1 ml aliquots were withdrawn and pipetted into 0·1 ml of cold preparation buffer. When all the heat-treated samples were ready, they were assayed by the standard procedure for NADH–NAR activity. Aliquots from untreated enzyme (cell-free preparation) samples served as controls.

EFFECT OF ENZYME INHIBITORS

In order to prepare a suitable buffer medium for maintanining the enzyme in a stable form after isolating from cells, sensitivity of the enzyme to various stabilizers and inhibitors was tested. The enzyme activity was inhibited by pHMB, cyanide, azide and EDTA very effectively (table 6). The inhibition of pHMB was largely overcome by the addition of DTT.

4. DISCUSSION

Nitrate reductase, reported to be soluble in *Neurospora*,[8] plants[24] and some bacteria,[25] and particulate in the yeast *H. anomala*[16] and some bacteria,[26] is apparently soluble in *C. utilis*.

Several enzymatic activities such as flavin-dependent NADPH-cytochrome *C* reductase, FNH_2-NAR, MVH-/BVH-NAR were reported to be associated with NADPH-NAR in nitrate-induced *Neurospora*[8]. Nitrate reductase from *C. utilis* displayed only the reduced pyridine nucleotide dependent activity, *i.e.*, NAD (P) H: nitrate oxidoreductase (EC 1.6.6.2), thus differing from NAR of *N. crassa*,[8] *H. anomala*[14] and *T. nitratophila*.[12] However, the cell free preparations were not examined for NAD (P) H-cytochrome *C* reductase activity.

Formate, lactate, pyruvate and NADH served as effective electron donors for NO_3-reduction in *Escherichia coli*.[27] However, of a wide range of reductants tested, only NADH and NADPH served as electron donors for NAR activity of *C. utilis* (table 3), suggesting a specific requirement of

Table 6. Effect of various inhibitors on *C. utilis* nitrate reductase activity *in vitro*

Inhibitor (1 mM)	NAR activity (% control)
Cyanide ..	2·9
Azide ..	18·6
EDTA ..	11·4
pHMB ..	1·4
DTT ..	62·1
pHMB + DTT	67·2

NADH–NAR activity was assayed *in vitro* at 37° C as described in the text. Enzyme preparation (equivalent to 1 mg protein) was incubated with the inhibitor for 5 min at room temperature before it (along with the inhibitor) was added to the reaction mixture.

a reduced pyridine nucleotide. *C. utilis* NAR, thus, resembled the enzyme from *H. anomala*[15] and plants[24] and differed from *Neurospora*[8] in its higher affinity for NADH over NADPH. The failure of added FAD to enhance NAR activity may be due to the endogenous flavin, tightly bound to the enzyme.

This enzyme had no latent activity as reported in the case of *N. crassa*[5] and was found to be unstable on storage and dialysis.[13]

The extreme sensitivity of the enzyme to cyanide, azide and EDTA suggests the involvement of a metal, shown to be molybdenum by earlier workers[28,29] functioning in the electron transfer during nitrate reduction, catalyzed by NAR (table 6).

The inhibitory effect of pHMB suggests the involvement of one or more relatively accessible sulfhydryl groups in the initial part of the electron transfer sequence (functioning possibly in the binding of the electron donor), catalyzed by NAR.

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ABBREVIATIONS USED :

NADH: reduced nicotinamide adenine dinucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide; FNH_2: reduced flavin nucleotide; DTT: dithiothreitol; EDTA: ethylenediaminetetracetic acid; pHMB: *p*-hydroxymercuribenzoate; MVH: reduced methyl viologen, and BVH: reduced benzyl viologen.

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Regulatory properties of yeast nitrate reductase *in situ*

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Regulatory properties of yeast nitrate reductase *in situ*[1,2]

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CHOUDARY, V. P., and G. R. RAO. 1976. Regulatory properties of yeast nitrate reductase *in situ*. Can. J. Microbiol. **22**: 35–42.

A simple and rapid procedure to make yeast cells permeable by agitating with toluene–ethanol, (TE) 1:4, v/v was developed. The permeated cells retained their ability to catalyze certain enzyme reactions. Temperature and duration of agitation during TE treatment played an important role in retention of the catalytic potential of permeated cells. The *in situ* assay using permeated cell preparations was more sensitive even in the absence of added cofactors than the in vitro assay in detecting assimilatory nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2) (NAR) activity in *Candida utilis*.

Using *in situ* assay technique, different mechanisms regulating the biosynthesis of NAR in *C. utilis* were investigated. Nitrogen starvation did not lead to derepression of NAR. NO_3^- ions were absolutely essential for induction and maintenance of high levels of NAR activity. Cells grown on ammonium nitrate possessed relatively lower levels of NAR. Kinetics of NAR induction were followed as a function of time and inducer concentration. The influence of various cations on the induction of NAR by nitrate was investigated. A wide range of D-amino acids induced NAR synthesis. Of 22 L-amino acids tested only phenylalanine induced significant levels of NAR. Various intermediates of the pathway of nitrate reduction influenced the rate of NAR induction. There was a rapid disappearance of in vivo activity of the enzyme of induced yeast cells on nitrogen starvation, and the rate of loss was accelerated by the presence of NH_4^+.

CHOUDARY, V. P., et G. R. RAO. 1976. Regulatory properties of yeast nitrate reductase *in situ*. Can. J. Microbiol. **22**: 35–42.

Une procédure simple et rapide pour rendre les cellules de levure perméables par agitation avec le toluene–éthanol (1:4, v/v) fut développée. Les cellules perméabilisées retiennent leur abilité à catalyser certaines réactions enzymatiques. La température et la durée de l'agitation au cours du traitement TE jouent un rôle important dans la retention du potentiel catalytique des cellules perméabilisées. L'essaie *in situ* utilisant des préparations de cellules perméabilisées est plus sensible même en l'absence de cofacteurs qui laissent in vitro pour détecter la nitrate réductase assimilatoire (NAD(P)H: nitrate oxidoréductase, EC 1.6.6.2) (NAR) chez *Candida utilis*.

En utilisant la méthode d'essaie *in situ* nous avons étudié différents méchanismes régularisant la biosynthesis de NAR de *C. utilis*. La privation d'azote ne conduit pas à la dérépression de NAR. Les ions nitrates sont absolument essentiels pour l'induction et la maintenance de hauts niveaux d'activités NAR. Les cellules qui se sont développées sur le nitrate d'ammonium possèdent relativement de faibles niveaux de NAR. Les kinétiques de l'induction du NAR furent suivies en fonction du temps et la concentration de l'inducteur. L'influence de cations variés sur l'induction du NAR par le nitrate fut aussi étudiée. Une grande variété de D-acides aminos induit la synthèse du NAR. Des 22 L-acides aminés essayés, seulement la phénylalanine induit des niveaux significatifs du NAR. Des intermédiaires variées du chemin méthabolique de la réduction du nitrate influencent le taux de réduction du NAR. Il y a une disparition rapide de l'activité in vivo de l'enzyme induite chez des cellules de levure sur une diette d'azote, et le taux de disparition est accéléré par la présence d'amonium.

[Traduit par le journal]

Introduction

Nitrate reductase (NAR), which catalyzes the reduction of nitrate to nitrite, is the 'first' enzyme in the pathway of nitrate assimilation. Its regulation and properties were extensively investigated in plants (3), algae (20), fungi (2, 31), and bacteria (8). Despite the fact that the ability to assimilate nitrate formed a chief criterion in the identification and classification of yeasts (36), little was known of the physiological and biochemical aspects of nitrate metabolism in yeasts (27) with the exception of a few preliminary reports on NAR by Silver (29), Pichinoty and Mêtênier (24), Rivas *et al.* (27), and Choudary

[1]Received April 28, 1975.

[2]A preliminary account of a portion of this study was presented at the 15th Annual Meeting of the Association of Microbiologists of India, December 19–21, 1974, Bangalore, India.

[3]Recipient of a Junior Research Fellowship of the University Grants Commission, New Delhi, 1970 to 1973, and presently Senior (Predoctoral) Research Fellow of the Indian Institute of Science, Bangalore, India.

and Rao (4). It was therefore of great interest to study the regulatory mechanisms of NAR in yeast, particularly in various species of *Candida*, which vary widely in their capacity to assimilate nitrate.

Several ingenious methods were developed to assay enzymes *in situ* in permeated cells of bacteria (26), yeasts (28), and in intact nodulated roots (13) and leaf discs (25). The present paper reports an improved method to permeate cells of *C. utilis*, and the results of *in situ* experiments on kinetics of induction, repression, and in vivo stability of yeast NAR (NAD(P)H : nitrate oxidoreductase, EC 1.6.6.2).

Materials and Methods

Chemicals

NADH,[4] NADPH, FAD, and L- and D-amino acids were obtained from Sigma Chemical Co., St. Louis, U.S.A.; sulfanilamide from Indian Drugs and Pharmaceuticals Ltd., India; and *N*-(1-naphthyl)-ethylenediamine dihydrochloride from B.D.H. Ltd., Poole, England. Other chemicals were of analytical or reagent grade from B.D.H. Chemicals Division, Glaxo Labs. (India) Ltd., Bombay; Sarabhai M. Chemicals, Baroda, India, or Riedel-De Haenag Seelze-Hannover, Germany.

Organism, Media, and Growth Conditions

Candida utilis CBS 4511 was obtained from the Centraalbureau Voor Schimmelcultures, Delft, The Netherlands. The basal medium without nitrogen source was essentially as described by Wickerham (36) with a few modifications, and contained in addition, either of the nitrogen sources: ammonium sulfate, 150 m*M*(N), 'ammonium medium'; or potassium nitrate, 50 m*M*(N), 'induction medium.' The organism was maintained on medium solidified by the addition of 2% agar (Difco). The yeast was cultured in liquid medium in 500 ml Erlenmeyer flasks on an Emenvee gyratory shaker (180 rpm) at 30 °C.

Induction of NAR

Cultures grown till late exponential phase in ammonium medium were harvested, washed, transferred to the induction medium, and shaken for 4 h at 30 °C.

Preparation of Cell-free Extracts

Cells were washed, packed, and ground with three times their weight of carborundum for 15 min at 4 °C and extracted with three volumes of preparation buffer (0.1 *M* potassium phosphate buffer, pH 7.0, with 1.5×10^{-4} *M* EDTA and 10^{-4} *M* DTT). The supernatant obtained by centrifugation of the slurry at 20 000 × *g* for 20 min in a refrigerated RC2-B Sorvall centrifuge was used to assay NAR activity in vitro.

Permeation of Cells

Washed yeast cells were suspended in preparation buffer to give 100 mg (wet wt.)/ml. An aliquot of 0.2 ml of chilled toluene–ethanol (TE), 1:4, (v/v) was added to 2 ml of the cell suspension in a 16-mm test tube, and shaken for 2 min on a Vortex mixer at 0 °C. Suitable aliquots of this preparation were used to assay NAR *in situ*.

Enzyme Assays

1. NAR in Vitro

The reaction mixture in a final volume of 1 ml contained sodium pyrophosphate buffer, pH 7.0, 0.04 *M*; KNO_3, 0.02 *M*; FAD, 0.01 m*M*; NAD(P)H, 0.10 m*M*; and 0.1 ml of enzyme (≃ 1 mg protein). The reaction was carried out at 37 °C for 10 min, terminated by the addition of 1 ml of 1% (w/v) sulfanilamide in 3 *N* HCl, and the nitrite formed was estimated according to Snell and Snell (30).

2. NAR in Situ

The reaction mixture in a total volume of 1 ml contained sodium pyrophosphate buffer, pH 7.0, 0.04 *M*; KNO_3, 0.02 *M*; and 0.5 ml (50 mg fresh weight) of permeated cell preparation. The reaction was carried out, terminated, and the nitrite content in an aliquot of the supernatant obtained after low-speed centrifugation was determined as described under the in vitro method.

Units and Specific Activity

One unit of the enzyme is equivalent to that amount which catalyzes the formation of 1 nmol of nitrite per minute, and the specific activity is expressed as units per milligram protein. The specific activity in the case of permeated cells is expressed as units per milligram of total extractable protein (55 mg/g fresh yeast).

Analytical Assays

Protein was estimated by the method of Lowry as modified by Hartree (10), with crystalline bovine serum albumin as standard. The nitrite content of 0.1-ml reaction mixture was determined by the method of Snell and Snell (30) with the addition of 1.0 ml of 1% (w/v) sulfanilamide in 3 *N* HCl, followed by 1.0 ml of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride, and distilled water to make up the final volume to 5.0 ml. After 15 min, the color intensity was read at 540 nm in a Beckman model DU spectrophotometer.

Results

NAR Activity in Permeated Cells of C. utilis

To detect NAR *in situ*, yeast cells grown till late exponential phase in induction medium were harvested, washed, and suspended in 0.1 *M* potassium phosphate buffer, pH 7.0, to give 10 mg (wet wt.) of cells/ml. Chilled TE (50 µl) was added to 1.0 ml of cell suspension and subjected to uninterrupted vigorous shaking for 5 min on a Vortex mixer at 30 °C. The cells thus permeated according to an earlier method (28) failed to show NAR activity *in situ*, indicating that either the cells were not sufficiently permeated to facilitate the entry of added nitrate, or

[4] ABBREVIATIONS USED: NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavine adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

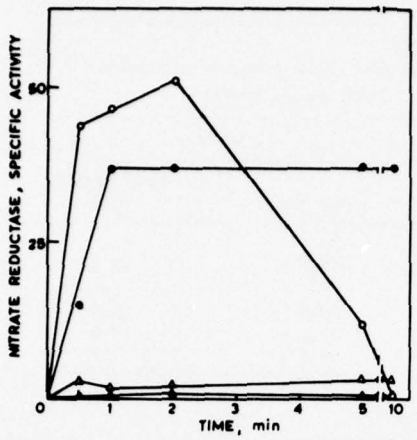

FIG. 1. Effect of temperature and mixing on permeation of *C. utilis* cells. NAR was assayed *in situ* as described in the Materials and Methods section. (●), TE addition followed by mixing at 0 °C; (○), TE addition followed by mixing at 30 °C; (△), TE addition at 30 °C, no mixing; (▲), TE addition at 0 °C, no mixing.

the conditions of treatment resulted in inactivation of the enzyme. This necessitated standardization of the conditions of permeation as depicted in Fig. 1.

Chilled TE (0.2 ml) was added to each of 2.0-ml aliquots of induced yeast cell suspension (100 mg (wet wt.) of cells/ml) in 16-mm tubes, and shaken for 0, 0.5, 1, 2, 5, and 10 min on a Vortex mixer at 0 °C or 30 °C, as indicated. These preparations were examined for NAR *in situ*. As Fig. 1 illustrates, whether at 0 °C or 30 °C, agitation was essential to make the enzyme accessible to the substrate. Although agitation at 30 °C resulted in rapid permeation initially, prolonged mixing beyond 2 min resulted in rapid loss of the enzyme activity. On the other hand, treatment at 0 °C, while making the cells permeable within 2 min, did not affect the enzyme activity even after extended periods of mixing. Agitating cells in the absence of TE at both temperatures failed to make cells permeable. The permeated cell preparations were stored at 0 to 4 °C up to 48 h without appreciable loss of NAR activity.

However, when the TE-treated cells were frozen at liquid nitrogen temperature and thawed, repeated twice, considerable amount of proteins appeared in the soluble (20 000 × *g* supernatant) fraction, which showed higher specific activity of NAR than the cell pellet (data not shown). This procedure facilitated isolation of enzymes with greater ease and minimum risk of inactivation unlike the conventional procedures for microbial cell disruption.

NAR Activity in Situ and in Vitro

To determine the relative efficacy and sensitivity of the *in situ* and the in vitro methods of assaying NAR, yeast cells grown in induction medium were washed and divided into two batches. One batch was treated with TE and examined for NAR *in situ*. The second batch was used to prepare the cell-free extract (termed 'crude preparation') which was divided into two parts. One part was left as such at 0 to 4 °C, and the second was passed through Sephadex G-25 column, equilibrated with preparation buffer, and both fractions were examined for NAR activity in vitro. The specific activity of NAR was 2- to 2.5-fold higher *in situ* than in vitro (Table 1). In the absence of added cofactors, the enzyme failed to catalyze the reaction in vitro. Addition of an electron donor, either NADH or NADPH, appeared more essential than FAD (Table 1). The enzyme showed partial loss of activity on gel filtration, and total loss on heat treatment for 3 min at 49 °C, indicating its labile nature.

For its greater sensitivity and non-requirement of added cofactors, the *in situ* method was used in regulatory studies of yeast NAR.

Influence of Nitrogen Source on the Induction of NAR

The yeast cells starved of nitrogen or grown on ammonium sulfate failed to show any NAR activity. However, the cells exposed to nitrate showed the highest specific activity (Table 2). NH_4NO_3 induced only low levels of NAR, and α-amino-*N*-butyrate just minimal levels. All other compounds tested failed to induce NAR.

Time Course of NAR Induction

NAR activity began to appear shortly after the addition of the inducer, and the specific activity rose to significant levels within 30 min without appreciable increase in cell mass (Fig. 2). The lag period preceding the appearance of NAR activity was very short unlike that in *Neurospora crassa* (15) and *Ustilago maydis* (17).

Dependence of NAR Induction on Nitrate Concentration

The induction was maximal with KNO_3 at

TABLE 1. Relative efficacy of *in situ* and in vitro assay methods in detecting nitrate reductase activity in *C. utilis*[a]

Assay system[b]	NAR specific activity		
		In vitro	
	In situ[c]	Crude S-20	G-25 filtered S-20
No added cofactors	26.2	—	—
Complete (NADH + FAD)	39.7	19.2	15.2
NADH omitted	29.7	—	—
FAD omitted	31.2	14.6	14.0
Complete (NADPH + FAD)	30.2	7.6	7.4
FAD omitted	26.4	4.4	5.3

[a]The yeast was grown for 12 h at 30 °C on 0.5% (w/v) KNO_3.
[b]The assay system was as described in the text.
[c]Specific activity expressed as units per milligram of total protein.
NOTE: —, Not detectable.

TABLE 2. Induction of nitrate reductase in *C. utilis* cells grown on various nitrogen sources

Nitrogen source[a]	NAR[b] sp. act.
Nitrogen starvation	—
Ammonium sulfate	—
Potassium nitrate	31.1
Ammonium nitrate	13.7
Urea	—
α-Amino-*N*-butyrate	3.2
Casein hydrolysate	—
Neopeptone	—
L-Asparagine	—

NOTE: Ammonia-grown *C. utilis* cells were transferred to induction media with different nitrogen sources, replacing KNO_3 shaken at 30 °C for 12 h, and the NAR activity assayed *in situ*. —, Not detectable.
[a]Concentration, 0.5% (w/v).
[b]Assayed *in situ*.

50 m*M*(N), and was less at lower concentrations (Table 3) showing a variance from the situation in *N. crassa* where only a low level of nitrate was required to enhance the capacity to synthesize NAR (33).

Influence of Cations on Induction of NAR

Sodium and potassium nitrates supported growth of yeast cells equally well and induced maximal levels of NAR. Magnesium and calcium nitrates induced only low levels of NAR although they permitted growth to occur (Table 4). Mercuric nitrate blocked the enzyme synthesis completely, but did not interfere with growth. Lithium, ferric, zinc, and manganous nitrates allowed induction of marginal levels of NAR without permitting growth of cells while cobaltous, lead, cupric, and silver nitrates inhibited growth as well as enzyme formation (Table 4).

Induction of NAR by L- *and* D-*Amino Acids*

Of 22 L-amino acids tested, L-phenylalanine induced NAR in significant levels, and L-asparagine, L-aspartic acid, and L-threonine induced marginal levels (Table 5). The other L-amino acids could not induce NAR. On the contrary, all the 15 D-amino acids tested induced NAR activity to varying extents. D-norleucine, D-threonine, and D-serine induced relatively high levels, followed by D-aspartic acid, D-isoleucine, D-valine, D-leucine, and D-histidine, in that order. In general, monoamino monocarboxylic, hydroxy-monoamino monocarboxylic, monoamino dicarboxylic, heterocyclic, aromatic, and monoamino dicarboxylic amino acids with amides, in that order, were better inducers than the sulfur-containing ones (Table 5). Except L-cysteine, L-threonine, and D-cystine, none of the amino acids inhibited growth.

Repression of NAR

The influence of different nitrogen metabolites related to nitrate reduction on NAR induction by nitrate was examined (Table 6). NH_4^+ repressed the synthesis of NAR by 14.7% at 10 m*M*(N), and by 37.7% at 50 m*M*(N). Nitrite, hydroxylamine, and hydrazine showed a high degree of repression even at 10 m*M*(N) concentration, while urea and glutamate repressed the enzyme by more than 50% only at 50 m*M*(N) concentration.

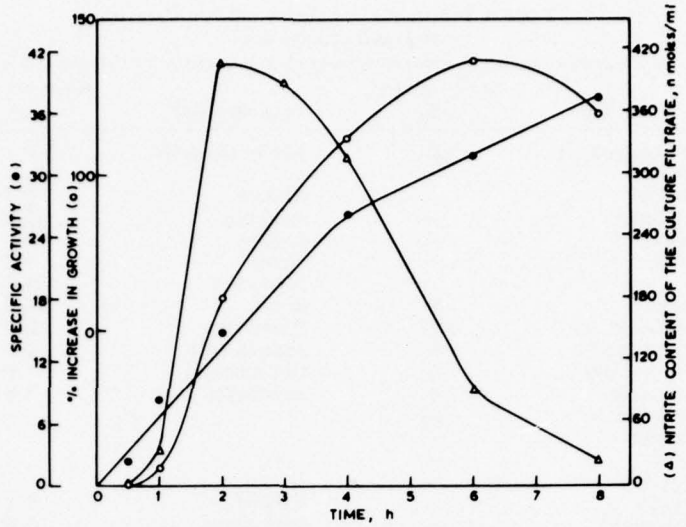

FIG. 2. Time course of nitrate reductase induction in *C. utilis*. NH_4^+-grown yeast cells were transferred to induction medium and shaken at 30 °C. Aliquots were removed at different intervals of time and nitrite content of the culture filtrate determined. NAR activity of washed cells was assayed *in situ*. (○), growth rate in terms of increase in cell mass (wet wt.) during induction; (●), NAR specific activity; (△), nitrite content of the culture filtrate, nmoles NO_2/ml.

TABLE 3. Effect of nitrate concentration on induction of nitrate reductase in *C. utilis*

Potassium nitrate concn., m*M*(N)	NAR[a] sp. act.
0.00	—
0.01	6.9
0.10	7.3
1.00	13.3
10.00	27.6
50.00	50.6
100.00	49.5

NOTE: The yeast cells were induced with different concentrations of KNO_3. All other details as under Table 2.
[a]Assayed *in situ*.

TABLE 4. Influence of cations on induction of nitrate reductase in *C. utilis* by NO_3^-

Nitrogen source[a] (*nitrate of*)	Increase in *wet wt.*[b], *g*	NAR[c] sp. act.
K^+	0.15	33.0
Na^+	0.15	33.0
Li^+	—	6.5
Ca^{2+}	0.15	16.5
Mg^{2+}	0.20	20.0
Mn^{2+}	—	1.5
Co^{2+}	—	—
Zn^{2+}	—	3.0
Fe^{3+}	0.05	5.4
Pb^{2+}	—	—
Cu^{2+}	—	—
Ag^{2+}	—	—
Hg^{2+}	0.20	—

[a]Induction was carried out as described in the text. KNO_3 was replaced with 0.5% (w/v) of the appropriate nitrate, wherever necessary.
[b]The initial wet weight of the cells used for induction was 0.4 g.
[c]Assayed *in situ*.
NOTE: —, Not detectable.

In Vivo Stability of NAR

The presence of nitrate not only stabilized in vivo NAR activity but also resulted in its enhancement. Nitrogen starvation as well as the presence of NH_4^+ led to about 50% loss in NAR activity within 1 h, and the rate of the loss after 2 h was much higher in the presence of NH_4^+ (Table 7).

Discussion and Conclusions

In making cells of yeast and bacteria permeable, the use of chelating agents (16, 19), polyene antibiotics (5), dimethyl sulfoxide (1), and toluene (11, 23, 28) is well documented. Among these agents, treatment with toluene found extensive use since it allowed the treated cells to retain considerable metabolic potential (11) while rendering them freely permeable to a variety of

TABLE 5. Induction of nitrate reductase in *C. utilis* by L- and D-amino acids

L-Amino acid[a]	NAR[c] rel. act., %	D-Amino acid[b]	NAR[c] rel. act., %
KNO_3[a] (control)	100	KNO_3[a] (control)	100
Glycine	—		
Alanine	—	Alanine	2.5
Isoleucine	—	Isoleucine	10.9
Leucine	—	Leucine	8.7
Valine	—	Valine	9.0
		Norleucine	27.8
Serine	—	Serine	16.5
Threonine[d]	0.5	Threonine	18.0
Aspartic acid	1.6	Aspartic acid	12.0
Glutamic acid	—	Glutamic acid	5.0
Asparagine	2.4	Asparagine	7.6
Glutamine	—		
Arginine	—		
Lysine	—		
Cysteine[d]	—		
Cystine	—	*Cystine*[d]	3.8
Methionine	—	Methionine	3.6
Phenylalanine	9.0	Phenylalanine	7.4
Tyrosine	—		
Tryptophan	—	Tryptophan	7.4
Histidine	—	Histidine	8.4
Proline	—		
Hydroxyproline	—		

[a]Concentration, 7 m*M*.
[b]Concentration, 3 m*M*.
[c]Assayed *in situ*.
[d]Failed to support growth.
NOTE: —, Not detectable.

TABLE 6. Repression of nitrate reductase in *C. utilis* by various nitrogen metabolites

Addition[a]	Concn., m*M*(N)	Increase in wet wt.[b], g	NAR[c] rel. act., %
None (control)	0	0.80	100
Potassium nitrite	10	0.20	10.9
	50	0.10	1.4
Hydroxylamine·HCl	10	0.10	7.1
	50	0.05	6.8
Hydrazine·H_2SO_4	10	0.10	1.9
Ammonium sulfate	10	0.20	85.3
	50	0.30	62.3
Glutamic acid	10	0.35	97.6
	50	0.25	45.4
Urea	10	0.65	91.5
	50	0.40	18.0

[a]The induction media were as described in the text. They contained different *N*-metabolites in the concentrations mentioned in addition to 0.5% (w/v) KNO_3; control had no addition.
[b]The initial wet weight of the cells before induction was 0.4 g.
[c]Assayed *in situ*.

substances (23). In the light of these reports, we developed an improved method to assay yeast NAR *in situ* (Fig. 1), which was more sensitive and simple than the in vitro method (Table 1). The consistently lower specific activity found in cell-free preparations passed through Sephadex G-25 supported the earlier report of the highly unstable nature of NAR in yeasts (27).

Candida utilis NAR was similar to that of *Hansenula anomala* (29) in its preferential

TABLE 7. In vivo stability of nitrate reductase in *C. utilis*

Duration of exposure, h	NAR[a] relative activity, %		
	KNO_3	No nitrogen source	$(NH_4)_2SO_4$
0	100	100	100
1	114.6	50.4	54.2
2	125.9	50.4	54.2
3	138.8	50.4	43.8
4	159.2	50.4	29.2
6	152.5	34.6	20.4
8	151.7	34.2	17.1

NOTE: The procedure of induction was as described in the text. Nitrogen source was KNO_3, 50 m*M*(N); or $(NH_4)_2SO_4$, 150 m*M*(N).
[a]Assayed *in situ*.

affinity for NADH over NADPH (Table 1). The yeast NAR thus closely resembled plant NAR and differed from the *Neurospora* enzyme which was reported to have a marked specificity for NADPH.

In *Chlorella vulgaris*, *Ankistrodesmus braunii*, and *Platymonas tetrathele*, a substantial increase in NAR activity after nitrogen starvation of NH_4^+-grown cells was reported (14, 20, 34). However, in *C. utilis*, nitrogen starvation did not result in the appearance of NAR activity. Thus, the NO_3^--dependent NAR induction in *C. utilis* resembled regulatory system in *Lemna minor* (21) and *N. crassa* (31), and its overall regulation operated through induction–repression (12) but not derepression–repression.

Candida utilis cells exposed to NH_4NO_3 retained significantly high levels of NAR activity suggesting a relatively lower degree of NH_4^+ antagonism unlike that reported in fungi (22) and yeasts (29), where NH_4^+ repression of enzymes involved in nitrogen utilization is of widespread occurrence (2, 6, 32). The role of NH_4^+ ions, in addition to causing repression, was also implicated in inactivation (18), degradation (17, 32), inactivation–repression (18), and regulation of uptake of NO_3^- ions (9). In *C. utilis*, NAR biosynthesis is regulated through repression not only by ammonia but also by other intermediates of nitrate assimilation to a great extent (Table 6).

The present results (Table 7) indicate that the repression–inactivation phenomenon common to yeasts (7) is also operative in the regulation of NAR in *C. utilis*, wherein the loss of enzyme activity caused by NH_4^+ was enhanced by an accompanying inactivation of the preformed enzyme as reported in *Chlorella* (20). NH_4^+ was shown to promote in vivo inactivation of NAR by reduction in *Chlamydomonas* (18), by degradation in *U. maydis* (17) and cultured tobacco pith cells (37), and by a protease action in maize roots (35).

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MOLECULAR BASIS OF NITRATE REDUCTASE INDUCTION IN CANDIDA UTILIS

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SUMMARY

Temporal separation of transcription and translation during nitrate reductase induction in Candida utilis was achieved by the use of actinomycin D and cycloheximide. The yeast failed to synthesize nitrate reductase when nitrate was not provided during transcription. Nitrate thus appeared to induce during transcription the capacity to synthesize nitrate reductase. Presence of nitrate, on the other hand, was not obligatory during translation except for its essential role in maintaining the stability of nitrate reductase after its formation as well as its mRNA.

The regulation of assimilatory nitrate reductase [NAD(P)H: nitrate oxidoreductase EC 1.6.6.2] has been the subject of intensive investigation in various organisms (2,4-9,11). In most of the systems investigated hitherto, nitrate reductase synthesis has been shown to require nitrate as inducer (9,2). Using different combinations of cycloheximide and actinomycin D, Sorger and Davies (9) demonstrated that NO_3^- ions are essential for the successful translation of NADPH-nitrate reductase mRNA species but not for the transcription of the nitrate reductase gene(s) in Neurospora.

We report in this communication the induced transcription-dependent synthesis of nitrate reductase in the yeast Candida utilis, and discuss the findings on this important variation in comparison with the other relevant literature reports.

MATERIALS AND METHODS

Chemicals: Actinomycin D and cycloheximide were purchased from Sigma Chemical Co., St. Louis, U.S.A. Rest of the chemicals,

apart from those described earlier (2), were all of analytical grade and obtained from either BDH (INDIA) Ltd., or from Sarabhai M. Chemicals, Baroda, India.

Organism and maintenance: C. utilis CBS 4511, obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, was used in all the experiments. The media, and procedures for maintenance and growth of the yeast and for induction of nitrate reductase were as described earlier (3).

Permeation of cells and assay of nitrate reductase: The procedures followed to permeate induced cells of C. utilis to assay nitrate reductase activity in situ and for analytical assays, and the definitions of enzyme units and specific activity were as described previously (2,3).

RESULTS

Effect of actinomycin D and cycloheximide: In inducible enzyme systems in bacteria, the inducer is known to enhance the enzyme levels by boosting the synthesis of the specific mRNA. An inducible enzyme can be synthesized from the preformed mRNA even if simultaneous transcription is blocked, and also in the absence of the inducer, provided the mRNA is stable enough. Basing on this fact, Turner et al. (12) devised a simple technique to achieve temporal separation of transcription and translation during induction of kynureninase in Neurospora and then continuing the incubation in inducer-free minimal medium without the drug.

Adopting a similar procedure to separate transcription and translation during nitrate reductase induction in C. utilis, an attempt was made to identify the specific step of the enzyme synthesis during which nitrate, as inducer, exerts its influence on the overall regulation of nitrate reductase induction.

Table 1. Effect of actinomycin D and cycloheximide on nitrate reductase induction in C. utilis

Antibiotic included in minimal medium	Final concentration (mg/ml)	Preincubation with inhibitor (min)	Addition of inducer[a]	Continued incubation period (min)	Nitrate reductase specific activity
A. Nil (Control)		30	+	120	19.0
B. Actinomycin D[b]	0.05	30	+	120	*
C. Cycloheximide	1.00	30	+	120	*
	5.00	30	+	120	*
	10.00	30	+	120	*
D. A portion of cells from system 'C', washed free from drug and inducer, and on continued incubation			-	120	6.3

Ammonia-grown cells were exposed to the drug in minimal medium for 30 min at 30°C. Then 1% (w/v) KNO_3 was added and shaking was continued at 30°C. After 2 h, an aliquot of each culture was drawn, washed and examined for nitrate reductase activity *in situ*. Cells from an aliquot of culture 'C' (exposed to cycloheximide, 1 mg/ml) were washed and transferred to fresh minimal medium without the drug as well as inducer, and the shaking at 30°C was continued for additional 2 h, at the end of which they were examined for nitrate reductase activity.

[a] KNO_3, 50 mM(N).

[b] The uptake of actinomycin D by the cells was facilitated by inclusion of 0.1% (v/v) toluene in the medium.

+ present; - absent; * not detectable.

Actinomycin D, known to prevent DNA-dependent RNA synthesis in eukaryotes as well as in prokaryotes, at 50 μg/ml concentration, completely inhibited induction of nitrate reductase in C. utilis. A relatively high concentration of actinomycin D was used in view of the earlier reports on fungi (6,10). Similarly, cycloheximide, a potent inhibitor of translation, at all concentrations ranging from 1 to 10 mg/ml, effectively blocked nitrate reductase induction (Table 1).

However, cells exposed to nitrate in presence of cycloheximide, after removal of the drug and inducer followed by incubation for 2 h at 30°C in fresh minimal medium, showed considerable levels of nitrate reductase activity (Table 1). On the contrary, the cells exposed to actinomycin D, after similar procedure, failed to show any nitrate reductase activity.

DISCUSSION

The total failure of nitrate in the presence of actinomycin D to induce nitrate reductase in C. utilis indicates that nitrate reductase is synthesized de novo and that the induced synthesis of the enzyme is transcription-dependent. The cells exposed to nitrate in presence of cycloheximide had apparently accumulated the capacity to synthesize nitrate reductase which was translated in minimal medium once the drug was removed. These results suggest that the presence of nitrate is absolutely necessary only for formation of the specific mRNA but not necessary during translation of the mRNA. However, the fairly low level of nitrate reductase activity of these cells compared to those of the control ones indicates the high lability of nitrate-reductase-mRNA species and possibly of the enzyme also, as in the case of Neurospora (10,9); we have already reported that

nitrate reductase in C. utilis is rapidly inactivated in vivo in the absence of nitrate (2).

Thus, these results clearly establish that nitrate ions are required during transcription for accumulation of the capacity to synthesize nitrate reductase in C. utilis, contrary to the situation in Neurospora, where the presence of nitrate was reported to be essential for successful translation of nitrate reductase-mRNA type(s) but not for the transcription of nitrate reductase gene(s) (9). However, the important role of nitrate in maintaining the stability of nitrate reductase, after its formation, in C. utilis is analogous to the regulation of the enzyme in Neurospora (1).

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D. LIST OF PAPERS PRESENTED AT CONFERENCES

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   V. Prabhakara Choudary and G. Ramananda Rao (1972)
   Proc. Soc. Biol. Chem. (India), 31, 22.

2. Phenethyl alcohol and its effects on Candida species.
   T.K. Narayanan and G. Ramananda Rao (1972)
   ibid., 31, 23.

3. Miconazole and its action on Candida species.
   G. Ramananda Rao, K.H. Sreedhara Swamy and M.Sirsi (1972)
   ibid., 31, 24.

4. Dermatophytes from clinical samples and their sensitivity to antifungal compounds.
   Vijaya Manohar, G. Ramananda Rao, M. Sirsi and N. Krishna Murthy (1972)
   ibid., 31, 24.

5. Pathogenic yeasts in superficial mycoses.
   Vijaya Manohar, K.H. Sreedhara Swamy, G. Ramananda Rao and M. Sirsi (1973)
   Presented at the 14th Ann. Conf. Assoc. Microbiol. of India held at Poona. P-11 (in abstracts).

6. Studies on the mechanism of action of miconazole on Candida species.
   K.H. Sreedhara Swamy, G. Ramananda Rao and M. Sirsi
   ibid., p.77.

7. Effect of miconazole on sheep erythrocytes.
   K.H. Sreedhara Swamy and M. Sirsi (1974)
   Proc. Soc. Biol. Chem. (India) 33, 7.

8. Morphological and biochemical correlation of Microsporum canis spore germination.
B.M. Jayaram, K.H. Sreedhara Swamy and G. Ramananda Rao (1974)
ibid., 33, 31.

9. β-Indoleethanol and β-indolelactic acid production by Candida species: their antibacterial and auto-antibiotic action.
T.K. Narayanan (1974)
ibid., 33, 46.

10. Antigenicity of Epidermophyton floccosum.
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Presented at the 15th Ann. Conf. Assoc. Microbiol. of India held at Bangalore. P.11 (in Abstracts).

11. Regulatory properties of nitrate reductase in situ.
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G. Ramananda Rao, K.H. Sreedhara Swamy and M. Sirsi (1975)
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14. Regulation of phenylalanine aminotransferase activity in Candida guilliermondii.
Y. Gopalakrishna and G. Ramananda Rao (1977)
Paper accepted for presentation at 46th Annual General Meetings of Society of Biological Chemists (India) to be held at Madras, India, during September 22-25, 1977.

15. Resistance of Microsporum canis to cycloheximide.
B.M. Jayaram and G. Ramananda Rao (1977).
ibid.

16. Immunity in experimental dermatomycoses.
Vijaya Manohar and G. Ramananda Rao (1977)
ibid.

E. LIST OF BOOKS AND JOURNALS OBTAINED

Sl. No.	Name of the book	Author(s)	Publishers&Year
1.	Diagnostic Medical Mycology	L.D. Haley	Appleton-Century-Crafts, New York, 1964
2.	The Yeasts Vol.I)	A.H.Rose &	1969 Academic Press,
3.	The Yeasts Vol.II)	J.S.Harrison	1971 London, New York
4.	Methods in Microbiology-Vol.5B	J.R.Norris & D.W.Ribbons	Academic Press, London, New York, 1971
5.	Atlas of Medical Mycology	E.S.Moss & A.L.McQuown	The Williams and Wilkins Co., Baltimore
6.	The Chemistry and Biology of Yeasts	A.H. Cook	Academic Press, New York.
7.	Mycopathologia Et Mycologia Applicata - Vol.39)		W.Junk N.V., The Hague, The Netherlands 1969, 1971 & 1971
8.	,, - Vol.44)		
9.	,, - Vol.45)		
10.	Practical Laboratory Mycology	Koneman & Elmer, W.	
11.	Mycological diagnosis of animal dermatophytoses	J.Dvorak M.Otcenasek	W.Junk N.V., The Hague, The Netherlands.
12.	Immunology and skin proceedings of a symposium held at Salishenlodge, Gleanden Bea ctr Montagna (William) Billingham (Rupert E)		
13.	Essential Immunology	E.M. Roitt	Blackwell Scientific Publications, Oxford, London, Edinburgh, 1971

14.	Medical Mycology, 3rd Ed.	B.S.Beneka & A.L.Rogers	Burgess Publishing Company, Minneapolis, Minn.
15.	Systemic Mycoses Ciba Foundation Symposium	G.E.W. Wolstenholme & R.Porter	J.&A.Churchill Ltd., London, 1968
16.	Molecular Biology of gene, 2nd Ed.	J.D.Watson	W.A.Benzamin Inc. Menlopark, California,1970
17.	Dermatologic Differential Diagnosis	T.B.Fitz-Patrick & S.A.Walker	Year Book Medical Publishers Inc., Chicago, 1962
18.	Medical Mycology (2nd Ed.)	C.W.Emmons, H.B.Chapman & P.U.John	Lea & Febiger, Philadelphia, 1970
19.	The Year Book of Dermatology	F.D.Malkinson & R.W.Pearson	Year Book Publishers, Chicago, 1972
20.	Text Book of Dermatology Vol.I (2nd Ed.)	A.Roo, D.S. Wilkinson & F.J.G.E. Bling	Blackwell Scientific Publishers, Oxford, London, 1972
21.	,, Vol.II	,,	,,
22.	Fundamentals of Mycology	J.H.Burnett	Edward Arnold Publishers Ltd., Burnett, London 1968.

F. EQUIPMENTS OBTAINED

1. Shimadzu UV-Vis double beam spectrophotometer Shimadzu, Japan.
2. French Pressure Cell, American Instruments Co., U.S.A.
3. Titanium 50 rotor for ultracentrifuge, Beckman, U.S.A.
4. MINIRAC Fraction Collector, LKB Products, Sweden.
5. Binocular Research Microscope, Meopta, Model 3N 816, Czechoslovakia.
6. Acrylamide gel electrophoresis apparatus, Toshinwal, India.
7. Waring Blendor, Arthur H. Thomas Co., U.S.A.
8. Kelvinator Refrigerators (two numbers), Spencer and Co. Ltd., India.
9. Kelvinator Deepfreezer, India.
10. All glass distillation set, NPL, India.
11. pH meter PP 1040 type, PHILIPS, India.
12. Rotary Shaker, Model 33, Emenvee Engineers, India.
13. UV-inoculation chamber.

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